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## Genetic and Cytological Analysis of SPE-6, a *C. elegans* Tau Tubulin Kinase

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# Genetic and cytological analysis of SPE-6, a *C. elegans* Tau Tubulin Kinase

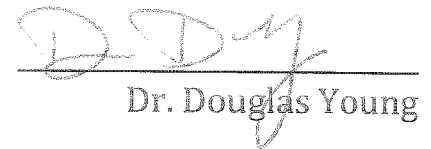
A thesis submitted in partial fulfillment of the requirements for the degree of  
Bachelor of Science with Honors in Biology at the College of William & Mary.

Jackson Jon Peterson

Accepted for Honors

A handwritten signature in cursive script, appearing to read "Diane Shakes", written over a horizontal line.

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Williamsburg, Virginia  
Spring 2015

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### **Contributions:**

Leah Towarnicky initially optimized the anti-SPE-6 antibody, preliminarily characterized the wild type localization of SPE-6, and performed the western blot in figure 1.

Brianna Waller helped with worm maintenance and genetics for some experiments.

Claire Tocheny performed western blots in figures 5 and 10 and quantitatively analyzed sperm motility in figure 7.

Craig Lamunyon at California State Polytechnic University, Pomona genetically isolated the *spe-6(hc187)* mutation from *spe-27(it110)* and performed the *spe-6(hc187); spe-29* self progeny experiment in figure 6.

Diane Shakes provided technical assistance, and participated in experimental design, data analysis, and in preparation of this thesis.



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**Abstract:**

To acquire motility, *Caenorhabditis elegans* sperm must undergo the regulated cellular remodeling process of sperm activation, but in the complete absence of new protein synthesis. Nematode sperm utilize two general mechanisms to overcome this obstacle: 1) They pre-package many proteins involved in this remodeling and subsequent sperm motility within the unique Fibrous Body-Membranous Organelle (FB-MOs) complexes; 2) They employ large numbers of protein kinases and phosphatases, including SPE-6, in the acquisition and regulation of motility. Genetically, *spe-6* is represented by two classes of alleles. In null alleles, spermatocytes fail to assemble FB-MOs or complete their meiotic divisions. In a second class of special alleles, spermatocytes complete their meiotic divisions, but mutant sperm activate precociously in the absence of proper extracellular signals.

In this study, we contextualize SPE-6 evolutionarily by demonstrating that SPE-6 is closest in sequence homology to Tau Tubulin Kinases and that SPE-6 itself is highly conserved throughout Nematoda. We then report the first description of the dynamic sub-cellular localization pattern of SPE-6 and how this pattern changes at key steps of *C. elegans* sperm development. Specifically, SPE-6 localization dramatically shifts during disassembly of FB-MOs and during sperm activation. Furthermore, we characterize *spe-6* sperm activation alleles at the protein and phenotypic level to determine that mis-localization or partial loss of SPE-6 expression is sufficient to induce precocious sperm activation. We further examine SPE-6 localization under a number of genetic, pharmacological,

and physiological conditions and reveal that SPE-6 localization is context dependent in activated sperm. Finally, we demonstrate that SPE-6 localization changes during sperm activation correlate to a change in phosphorylation state.

## **Introduction:**

### ***C. elegans* spermatogenesis as a model:**

Among genetic model organisms, *C. elegans* possesses unique features facilitating the identification of sperm specific cellular processes. The *C. elegans* gonad makes up nearly half the mass of an adult animal and is easily extruded for cytological studies. Since a single gonad contains a linearly arranged developmental progression all the way from mitotic stem cells to differentiated mature gametes, cytological analysis of the entire progression of spermatogenesis can be analyzed in a single gonad. Furthermore, the final step of spermatogenesis, sperm activation, can be induced and studied *in vitro*, which allows one to view and study the intermediate steps of this rapid process (Figure I-1). Most importantly, quick generation times and robust genetic tools allow for sophisticated genetic analysis.

Because all of *C. elegans* spermatogenesis and oogenesis can occur in the common reproductive track of the self-fertile hermaphrodites (Figure I-2), genetic screens have been designed in *C. elegans* which specifically identify mutations that only affect sperm biology. In other systems, such as *Drosophila melanogaster*, genetic screens that are intended to isolated spermatogenesis-defective mutants must filter out many other phenotypes related to male fertility

(Castillon et al. 1993). In *Drosophila*, male sterile phenotypes include defects in mating behavior, gross anatomy, or male health generate the same organismal level phenotypes as defects in spermatogenesis. In *C. elegans*, spermatogenesis-specific mutants can be isolated in genetic screens for normally self-fertile hermaphrodites that instead lay unfertilized oocytes, but are still fertile when mated with a wild type male. Such screens have identified a number of “spe” (spermatogenesis defective) genes specifically involved in spermatogenesis (L’Hernault, 2006). These screens and the subsequent analysis of the resulting mutants have deeply informed our understanding of spermatogenesis and fertilization as well as fundamental processes in cell biology. This thesis will describe a cytological and genetic analysis of *spe-6*, a kinase encoding gene that was previously discovered in two distinct spermatogenesis-related genetic screens.

### **Brief overview of *C. elegans* spermatogenesis:**

*C. elegans* hermaphrodites are somatic females which produce sperm for a limited time during the beginning of their reproductive life. Individual hermaphrodites produce approximately 150 sperm in each of their two gonadal arms during the final larval stage (L4). Coinciding with the onset of adulthood, the hermaphrodite germline switches over to producing exclusively oocytes. In contrast, males begin producing sperm in their single gonadal arm during the final larval stage and continue to produce sperm throughout their reproductive lifetime.

In both males and hermaphrodites, the germ line is housed in a linearly arranged syncytial gonad. Cells are pushed distal to proximally as they undergo major developmental changes from mitotic daughter cells to meiotically produced haploid gametes. Briefly, spermatocytes detach from a syncytial bridge, called the rachis, during late meiotic prophase following an interesting sperm specific stage marked by super compacted chromatin known as the karyosome stage (Shakes et al., 2009). Staining for an active epitope of RNA polymerase II stops during the karyosome stage and for the rest of the steps of spermatogenesis, indicating that the subsequent meiotic divisions occur without production of new transcripts. Nuclear envelope breakdown and chromosome catching by the microtubule spindle occur quickly after primary spermatocytes detach from the rachis. Following metaphase and anaphase of meiosis I, spermatocytes undergo a partial or complete cytokinesis event to form two secondary spermatocytes. Metaphase and anaphase of meiosis II occur quickly afterwards and are followed by a polarization event and an asymmetric budding division which produces spermatids and a cellular waste bag known as a residual body (Figure I-1, “budding”). Following budding, spermatids undergo a maturation process involving sperm specific Fibrous Body Membranous Organelles (see below). Spermatids activate into motile spermatozoa upon entry into the spermatheca in hermaphrodites, or upon copulation and entrance into the hermaphrodite uterus in males.

#### **Fibrous-Body Membranous-Organelles:**

*C. elegans* sperm must overcome a unique challenge due to the absence of protein synthesis in spermatids. Rather than the burst of transcriptional and translational activity following the meiotic divisions seen in mammalian (Bettegowda and Wilkinson, 2010) or *Drosophila* (Fabian and Brill, 2012) sperm, all transcripts required for *C. elegans* sperm function must be formed prior to transcriptional turn-off in the karyosome stage, and all proteins must be synthesized before loss of ribosomes in the budding division (more below). For instance, major sperm protein (MSP), which drives motility of the crawling spermatozoa, is translated beginning in meiotic pachytene, but does not have a known functional role until sperm activation, which can occur days after the protein is first synthesized. To overcome this challenge, *C. elegans* uses a unique organelle complex called the Fibrous Body-Membranous Organelle (figure I-1, green and white). The FB is a filamentous structure composed mostly of polymerized MSP, although our lab has recently identified two additional components. The MO surrounding the FB is a Golgi-derived organelle that functions in part to package the FB for proper segregation to spermatids. The MO also packages and sequesters essential spermatozoa specific membrane proteins within spermatids and may function in ion homeostasis (Smith 2014).

FB-MOs are formed *de novo* during spermatogenesis, and appear to be essential to successful spermatogenesis and fertility. *spe-6*, *spe-18*, and *spe-44* are all required for fibrous body formation (Varkey et al., 1993; Shakes et al., 2012; unpublished data Kari Price and Diane Shakes). SPE-44 is a sperm specific transcription factor regulating expression of approximately one third of all

sperm specific genes, including many MSP genes and SPE-18; therefore the requirement for SPE-44 in FB-MO morphogenesis likely works through SPE-18. SPE-18 protein localizes directly to the FB-MO implying direct involvement of SPE-18 in the formation of FBs (Shakes et al., 2012). *spe-18* spermatocytes arrest in the meiotic divisions without dividing and with multipolar spindles around a single chromatin mass, leading us to believe that cytosolic MSP interferes with normal cell cycle dynamics.

### ***spe-6* null mutants:**

Sequence analysis of *spe-6* determined that it encodes a member of the Casein Kinase I (CK1) serine/threonine kinase superfamily which has homologs present in all organisms from humans to yeast (Muhlrad and Ward, 2002) (Figure I-3 for amino acid sequence). Two putative null alleles of *spe-6* have been described (Varkey et al., 1993). The first, *spe-6(hc49)*, encodes a nonsense mutation truncating much of the C-terminus of the protein, likely leading to nonsense mediated decay of the transcript (Figure I-3). The second, *spe-6(hc92)* or *spe-6(hc143)* (these two strains encode the same lesion) is a mutation in a conserved residue that stabilizes the kinase domain; the same mutation has been identified in a yeast Casein Kinase I as a null allele (Murakami et al., 1999).

*spe-6* null mutants fail to form fibrous bodies but appear to have morphologically normal MOs by TEM analysis (Varkey et al., 1993). The published *spe-6* terminal phenotype is a diakinesis arrested primary spermatocyte with four spindle poles around a central nucleus although studies



in our own lab suggest a slightly later pro-metaphase arrest and additional cytoskeletal defects (Leah Towarnicky, J. Peterson, and D. Shakes, unpublished data; this thesis – future directions).

In addition to these null mutants, a number of “special” non-null alleles of *spe-6* have been identified which proceed beyond the meiotic divisions to form spermatids but then exhibit a phenotype involving the acquisition of sperm motility. Analysis of these alleles and the involvement of SPE-6 in sperm motility will be the focus of this thesis.

### **Budding and spermatid maturation**

Following the meiotic divisions of the chromosomes and depending on whether the cytokinesis step following anaphase II is complete or incomplete, anaphase II is followed by a polarized budding division during which two or four haploid spermatids bud from a central residual body. These budding spermatids retain mitochondria and the specialized FB-MO complexes whereas a number of cytoplasmic components including actin, tubulin, and ribosomes are deposited in the central residual body (Figure I-1). Therefore, *C. elegans* sperm are incapable of protein synthesis. *C. elegans* sperm also lack the capability for actin or tubulin based motility. Instead, nematode sperm utilize a unique mechanism of motility based on a structural protein called the Major Sperm Protein (MSP) (Stewart and Roberts, 2012).

Following the budding division, spermatids undergo a process called spermatid maturation. FBs break down leaving only MSP dimers within the

cytosol (Chu and Shakes, 2012) (Figure I-1). The MOs persist and dock with but do not fuse to the spermatid plasma membrane. Both cytosolic MSP and the MOs are intimately involved in later steps during sperm activation (see below).

The *C. elegans* spermatid is a very interesting cell type that lies dormant in the male seminal vesicle until copulation. *C. elegans* spermatids remain transcriptionally and translationally silent and may be entirely quiescent; however, metabolic activity has not been assessed. The sperm chromatin is tightly condensed into a single mass by protamine like proteins that replace the core histones. This chromatin mass is not encapsulated by a nuclear membrane, but is instead surrounded by an electron dense, RNA rich perinuclear halo which may have functions in epigenetic inheritance (Conine et al., 2013; Wolf et al., 1997).

### **The acquisition of motility in nematode sperm:**

Following copulation for male sperm or entrance into the spermatheca for hermaphrodite sperm, sessile spermatids acquire motility in a process known as sperm activation (see Figure I-1 for sperm activation, Figure I-2 for reproductive anatomy). Sperm activation is generally defined by two cellular morphological changes: first, the MOs fuse with the cell membrane; and second, the cell polarizes and extends a large MSP based pseudopod. The membrane fusion event during sperm activation means that this process is to some extent irreversible, although it is clear that sperm are capable of partially retracting pseudopodia. The pseudopod is formed from the coalescence of a number of

MSP containing spikes during an intermediate stage of activation (Figure I-3 for schematic, Figure 9 of results for DIC image). In a similar manner to the actin-based motility of lamellipodia, the MSP-based motility of these pseudopods is driven by the dynamic polymerization/depolymerization of MSP filaments (Stewart and Roberts 2012) (discussed below). Since the haploid spermatids are translationally silent, post-translational modifications like phosphorylation must be relied on for the morphological and functional changes of sperm activation.

Within the seminal vesicle of males, sperm exist as inactivated spermatids but isolated male sperm can be activated *in vitro* by the addition of certain pharmacological agents, allowing exploration of physiological features of sperm activation (Shakes and Ward, 1989). Known activators include the weak base triethanolamine (TEA), the ionophore monensin, the stilbene chloride channel inhibitor 4,4'-diisocyanatostilbene-2,2'-disulfonic acid (DIDS), and various calmodulin inhibitors (Smith, 2014). The identity of these activators implicates ion homeostasis and pH in sperm activation and motility (Shakes and Ward, 1989; Stanfield and Ellis, 2014). In particular, intracellular calcium appears to play a role in sperm activation (Smith 2014). This role may be mediated in part by *fer-1*, a large structural protein required for proper MO fusion during sperm activation (Ward et al., 1981; Washington and Ward, 2006). Notably, not only does FER-1 contain calcium dependent phospholipid binding domains (C2 domains) but *fer-1* mediated vesicle fusion is also sensitive to calcium levels, representing a connection between calcium signaling and sperm activation.

In addition to early arrest mutants like the *spe-6* null alleles described above, *spe* screens have identified a set of genes required for sperm activation in hermaphrodites, and therefore hermaphrodite self-fertility (Figure I-4). This set of genes, collectively called the *spe-8* class, contains *spe-8*, *spe-12*, *spe-19*, *spe-27*, and *spe-29* (Shakes and Ward 1989; Minniti et al., 1996; Geldziller et al., 2005; Nance et al., 2000). Of this group, SPE-12, SPE-19, and SPE-29 are predicted to be novel transmembrane proteins; while SPE-27 is a small novel hydrophilic protein and SPE-8 is an SH2 domain containing non-receptor tyrosine kinase (Muhlrad et al., 2014). Intriguingly, SPE-8 is localized to the plasma membrane of quiescent spermatids, and this localization require all other members of the *spe-8* class (Muhlrad et al., 2014). This observation suggests that these other SPE-8 class proteins establish a complex required for SPE-8 localization to the plasma membrane (Muhlrad et al., 2014). Upon normal sperm activation, SPE-8 leaves the plasma membrane, but its subsequent localization is unknown due to imaging limitations in Muhlrad et al. Sperm with mutations in these *spe-8* class genes are still capable of activation by *in vitro* activators like TEA that act on ion homeostasis, but are unresponsive to *in vitro* activation with proteases (see below). In wildtype sperm activated by TEA, SPE-8 fails to leave the membrane, consistent with TEA acting downstream of the *spe-8* class; in other words, bypassing the requirement for SPE-8. Taken together these data suggest that the *spe-8* class proteins function to transduce an extracellular signal to intracellular effectors inducing sperm activation, likely through changes in ion homeostasis.

Suppressor screens have identified mutations in genes that bypass the requirement for *spe-8* class genes in hermaphrodite self-fertility (Muhlrad and Ward, 2002) (Figure I-5). Remarkably, the vast majority of suppressor alleles from this screen defined a single recessive complementation group with mutations in *spe-6*. Since *spe-6* is absolutely required for earlier steps in spermatocyte development (see above), none of these alleles restoring sperm function encoded a molecular null. Instead, all of the sequenced *spe-6* mutants contained missense amino acid changes mapping to various regions of both the protein sequence and protein structure. The prototypical *spe-6* suppressor allele, *spe-6(hc163)*, is a recessive suppressor of the *spe-8* pathway, and suppressed all genes in the pathway tested. Analysis of *spe-6(hc163)* crossed out of a *spe-27* background determined that *hc163* males always (see results) contain precociously activated sperm in their seminal vesicles, a phenotype seldom observed in wild type (Figure I-6 for comparison of phenotypes of male sperm in seminal vesicle from wild type, *spe-6* null mutants, and *spe-6\** precocious activation mutants). *spe-6(hc163)* was selected as the prototypical suppressor allele because of its high fertility in double mutants with *spe-27(it132ts)*. In contrast, other *spe-6* suppressor alleles restored self-fertility to *spe-27(it132ts)* hermaphrodites but with reduced brood counts. It is logical to deduce that reduced brood counts in *spe-6; spe-27(it132ts)* animals could result either from disruption of the early function of SPE-6 in meiotic progression or from incompletely penetrant induction of a precocious activation phenotype (the mode by which these *spe-6* alleles restore hermaphrodite self-fertility to *spe-8* class

mutants). Indeed, TEM-based ultrastructural studies suggested that *spe-6(hc163)* spermatocytes have minor defects in MO assembly, implying that these alleles may also interfere with the early requirement for SPE-6 within developing spermatocytes. Analysis of double mutants revealed that *spe-6(hc163)* restored fertility to all members of the *spe-8* group tested, implying that the physiological effect of this *spe-6* mutation is to generally induce precocious activation, bypassing the requirement for physiological activation and the *spe-8* gene group.

Although rare in comparison to the frequent isolation of *spe-6* alleles, two other genes have been identified in *spe-27* (*spe-8* class) screens for genetic suppressors of a loss of *spe-8* class activity (Gosney et al., 2008; Liao et al., 2013). Null alleles of *spe-46* and special, non-null alleles of *spe-4* also cause precocious sperm activation and suppress deficiencies in the *spe-8* pathway. *spe-46* encodes a novel seven pass transmembrane domain protein (Liao et al., 2013) whereas *spe-4* encodes a homolog of the transmembrane metalloprotease presenilin (Arduengo et al., 1998). Mutations in both these genes also cause defects in MO formation, further implicating this compartment in sperm activation. A mechanism for precocious sperm activation is hinted at in *spe-46* spermatids which often contain precociously fused membranous organelles, a feature normally only seen in activated spermatozoa. In one model, the protein products of genes identified in these suppressor screens may function together to inhibit sperm activation until receipt of an extracellular signal. Alternatively, precocious sperm activation in some of these suppressor alleles may simply be a byproduct

of slightly defective FB-MO morphogenesis and not representative of a bona fide signaling pathway (Stanfield and Ellis, 2014). Further complicating this analysis, while the *spe-46* suppressor mutant likely encodes a loss of function allele, it has not been demonstrated whether the *spe-6* and *spe-4* suppressor alleles cause a gain of function or loss of function effect.

### **Mechanism of motility in nematode sperm:**

Work on a distantly related parasitic nematode, *Ascaris suum*, has provided biochemical insight into the mechanism of nematode sperm motility (Stewart and Roberts, 2012; Smith 2014). Structural studies demonstrated that the MSP dimer is the smallest functional unit, and that oligomerization of apolar MSP dimers constitutes higher order structures; these higher order structures are responsible for the MSP containing fibers visible with TEM in spermatocyte FBs and spermatozoa pseudopods..

A system of MSP polymerization and de-polymerization has been reconstituted *in vitro* from *Ascaris* sperm extracts allowing the identification of a number of factors involved in MSP based motility. In this system, MSP filaments nucleate on membrane vesicles and propel the vesicle forward in an ATP dependent manner (Stewart and Roberts, 2012). MSP itself lacks nucleotide binding capabilities, thus MSP polymerization is accomplished through the effects of MSP binding proteins. MSP binding proteins are an essential feature of MSP based motility and must be differentially localized at the leading (anterior) and cell body (posterior) sides of the pseudopod. MSP polymerization organizing

protein (MPOP) is essential to polymerization and functions to recruit MSP Polymerization Activating Kinase (MPAK), a serine/threonine phosphorylating Casein Kinase 1, to the pseudopod (LeClaire et al., 2004; Yi et al., 2007). MPAK phosphorylates another key MSP-binding protein, the MFP2 cytoskeletal protein, to promote fiber assembly. Tyrosine phosphorylation of proteins at the leading edge (anterior) of the pseudopod is an important feature; since tyrosine phosphorylation typically regulates localization, it is likely that this is a mechanism for the establishment of the polarity necessary to drive unidirectional motility.

MSP disassembly in *Ascaris* extracts is accomplished by the PP2A serine/threonine phosphatase dependent action of a third structural protein, MFP3 (Yi et al., 2007). To simplify the mechanism of *Ascaris* sperm motility, MSP polymerization at the leading edge of the pseudopod is promoted by phosphorylation of MSP associated cytoskeletal proteins by the serine/threonine kinase MPAK while the depolymerization at the cell body side is promoted by the dephosphorylation of cytoskeletal proteins by PP2A phosphatase (Smith, 2014).

Importantly, very few genes have been genetically identified in *C. elegans* that specifically regulate sperm motility and MSP dynamics in pseudopods; this implies that many of the genes involved in this process are likely either 1) functionally redundant/present in multiple copy numbers or 2) involved in other essential cellular processes that would stop organism or sperm development before sperm are made.



### **Sperm activation is accomplished by extracellular signals:**

Two key pieces of evidence imply the existence of a second redundant pathway independent of *spe-8* class genes (Figure I-4). First, males with mutations in the *spe-8* group of genes retain fertility (Shakes and Ward 1989). Second, when wild type males are mated to certain sterile alleles of the *spe-8* group, some of the otherwise unresponsive hermaphrodite sperm are activated, presumably by a signal present in the male ejaculate. Some models have proposed that two separate redundant signaling pathways exist, each responding to a different extracellular signal supplied by the male or hermaphrodite soma.

Early observations found that *in vitro* exposure to a non-specific protease cocktail or activated trypsin are each sufficient to induce sperm activation (Shakes and Ward, 1989; Smith and Stanfield, 2009). The idea that proteolytic activity is involved in sperm activation was strengthened by the identification of a secreted serine protease, *try-5*, which is a putative endogenous sperm activator (Smith and Stanfield, 2011). TRY-5 is dispensable for fertility in both males and hermaphrodites, but ejaculate from *try-5* males is unable to cross activate *spe-8* hermaphrodite sperm. Furthermore, GFP-labeled TRY-5 is released by the male vas deferens during copulation giving it the correct spatial and temporal localization to be an endogenous activator. While mutations in either *try-5* and *spe-8* class genes alone fail to prevent male fertility, double mutant combinations are sufficient to completely prevent sperm function in both males and hermaphrodites. The absolute requirement for either TRY-5 or SPE-8 has led to

the assumption that these two proteins function in separate sperm activation pathways.

Proteolytic activity is further implicated in sperm activation by the identification of SWM-1 (Stanfield and Villeneuve, 2006). The SWM-1 protein contains two trypsin inhibitor-like domains suggesting it functions to inhibit a secreted protease. While wild type males typically contain only inactivated spermatids, mutants deficient for SWM-1 contain large numbers of precociously activated sperm in the male seminal vesicle, implicating SWM-1 in the inhibition of sperm activation (the likely location of expression of SWM-1 is noted in Figure I-2). The reason behind the active employment of a protease inhibitor to inhibit sperm activation in the male is clear from phenotypic analysis of *swm-1* males which are infertile and fail to transfer sperm to the hermaphrodite during copulation (Stanfield and Villeneuve, 2006). TYR-5 is the likely target of SWM-1, since mutant males with null alleles of both *swm-1* and *try-5* contain no activated sperm. Although present in hermaphrodites, *swm-1* is not required for hermaphrodite self-fertility, leading to a model where precocious activation of male sperm prevents copulatory sperm transfer to the hermaphrodite but fails to affect hermaphrodite sperm which require no such transfer event during which precocious sperm activation must be inhibited.

A recent study has proposed that cationic zinc is the endogenous activator operating in the hermaphrodite reproductive track (Liu et al., 2013). *In vitro*,  $Zn^{2+}$  is able to induce sperm activation in a modified sperm media. Furthermore, zinc is enriched in specific compartments associated with sperm activation; zinc is

sequestered in the membranous organelles and mitochondria of sperm and is released upon sperm activation. Extracellularly, zinc is enriched in the secretory cells in the males and in the spermatheca of hermaphrodites. However, it has not been demonstrated how high concentrations of extracellular zinc might affect sperm activation, as no proteins associated with zinc binding have been implicated in sperm activation. Perhaps  $\text{Zn}^{2+}$  functions to activate a metalloproteinase located on the surface of sperm cells, as  $\text{Zn}^{2+}$  is the most common metal cofactor for these enzymes (Nagase et al., 2005)(and my speculation).

Interestingly, a recently described sperm expressed protein is required for TRY-5 mediated sperm activation, but not for *in vitro* activation by  $\text{Zn}^{2+}$  (Fenker et al., 2014). The sperm of *snf-10; swm-1* mutant males fail to exhibit precocious activation, and *snf-10* is required for hermaphrodite sperm to respond to cross-activation by male seminal fluid. Furthermore, both males and hermaphrodites lacking both *snf-10* and *spe-8* genes are completely infertile. SNF-10 shows homology to solute carrier 6 transporter proteins, which can facilitate changes in ion homeostasis, but it is not yet clear whether SNF-10 functions as a transporter in this system (Putative model for sperm activation pathways in figure I-4).

#### **Further evidence for two sperm activation pathways:**

While TRY-5 is called the “male activator” in the literature, genetic experiments show that TRY-5 mediated activation in the seminal vesicle of a *swm-1* mutant is dependent on the *spe-8* “hermaphrodite” pathway (Stanfield and

Villeneuve, 2006). While all alleles of *spe-8* class genes fail to block male fertility, hermaphrodite sperm with some alleles of the *spe-8* pathway are completely unable to be cross-activated by male seminal fluid (Stanfield and Ellis, 2014).

What then is the fundamental difference between male and hermaphrodite sperm activation? Two possible scenarios might explain some of the confusing experimental results: Either male and hermaphrodite sperm are intrinsically different or the physiological environment where sperm activation occurs contributes to the differences between male and hermaphrodite sperm activation. Additional receptors only utilized by male sperm, a difference in dosage of receptors, or a difference in response to receptors may be intrinsic differences between the two sperm types. However, at the transcriptional level almost no genes are enriched in male but not hermaphrodite spermatogenesis (Reinke, 2004). Perhaps it is a factor of size; the density of receptors on the cell surface may differ between the larger male sperm and the smaller hermaphrodite sperm. The size difference may not normally play a role in activation, but might be especially evident in a sensitized genetic background. Alternatively, a dose response effect may be in play where male sperm are capable of activating in a *spe-8* background simply because upon copulation they are exposed to a higher dose of TRY-5 than experienced in the seminal vesicle or by hermaphrodite sperm. Regardless of the upstream events of sperm activation, it has been demonstrated that *spe-6* mutations bypass a requirement for *snf-10*, *try-5*, and the *spe-8* pathway, supporting a model where the physiological effect of mutating

*spe-6* operates within the cell downstream of these various external signals (Smith and Stanfield, 2009; Fenker et al., 2014; Muhlrads and Ward, 2002).

### **Evolutionary importance of sperm activation:**

The presence of fertilization competent self-sperm in a feminine worm is the defining characteristic of a hermaphroditic nematode. While *C. elegans* researchers often take nematode hermaphroditism as a given, recent phylogenetic evidence demonstrates that the trait of hermaphroditism in *C. elegans* was one of three such independent events in the evolutionary history of the *Caenorhabditis* genus (Kiontke et al., 2011). The repeated origin of hermaphroditism in *Caenorhabditis* highlights the ease with which such a change can occur in the highly simplified, linearly arranged gonad system used by nematodes. Genetic studies of the *Caenorhabditis* male-female species, *C. remanei*, demonstrated that genetic changes inducing 1) sperm formation and 2) sperm activation were necessary and sufficient for the acquisition of hermaphroditism, highlighting the ease with which such a dramatic change in reproductive mode can occur in this clade (Baldi et al., 2009). Recent analysis showed that the *spe-8* group of sperm activation genes are conserved in sequence and expression patterns throughout the *Caenorhabditis* genus, and that many aspects of sperm activation are similarly conserved (Wei et al., 2014). The regulation of sperm activation is therefore highly important evolutionarily.

### **SPE-6 as a Tau Tubulin Kinase:**

While initial characterization of *spe-6* classified it as a Casein Kinase 1, we have examined current sequence data to determine that *spe-6* is more specifically homologous to Tau Tubulin Kinase 1 and 2 in mammals (Figure I-3, Figure I-7). TTBKs were identified biochemically as modifiers of the microtubule dynamics regulator Tau and other function of TTBKs are only beginning to be explored (Ikezu and Ikezu, 2014). Yet, already a number of high profile papers have discovered intimate links between TTBKs and important developmental processes and disease states (Goetz et al., 2012; Liachko et al., 2014). TTBK1 is specifically expressed in neural tissue and is associated with Alzheimer's pathogenesis; specifically, TTBK1 phosphorylates Tau on residues involved in tau aggregate formation and genetic studies implicate mutations in TTBK1 with late onset Alzheimer's disease. TTBK2 expression and function is more universal; TTBK2 is expressed in a number of human tissues and in mice has an essential function in the ciliogenesis required for embryonic development (Goetz et al., 2012). Loss of function mutations truncating the C-terminus of TTBK2 and disrupting protein localization are the causal mechanism for spinocerebellar ataxia type 11, a neurodegenerative disorder (Houlden et al., 2007; Bouskila et al., 2011).

The identity of SPE-6 as a relative to the Tau Tubulin Kinase (TTBK) members of Casein Kinase 1 offers some insight into SPE-6 function in the context of *C. elegans* sperm. Only three substrates of Tau Tubulin Kinases have been identified: Tau, the TDP-43 transcriptional regulator involved in Amyotrophic Lateral Sclerosis (ALS), and the CEP-164 protein involved in ciliogenesis (Ikezu

and Ikezu, 2014; Liachko et al., 2014). None of these targets are likely to be the target of SPE-6 in *C. elegans* sperm. Substrate specificity is thought to be determined by the C-terminus of TTBKs, of which SPE-6 has a highly divergent sequence (Čajánek and Nigg, 2014; Ikezu and Ikezu, 2014)(Figure I-3). Intriguingly, biochemical studies of TTBK2 have identified an unusual substrate preference for a phosphotyrosine residue near the site of phosphorylation, implying that TTBKs may act to modulate the activity of proteins already regulated by tyrosine phosphorylation (Bouskila et al. 2011).

Kinases and phosphatases are highly abundant in the *C. elegans* genome as compared to other organisms (Manning, 2005). Highly enriched are TTBK relatives, with over 30 present in the *C. elegans* kinome as compared to two in mice and humans. Transcriptomic studies have found that most of these TTBK members are unregulated in spermatogenesis (Reinke et al., 2000). Because of this abundance of related proteins, it is surprising that SPE-6 is required for early spermatogenesis and is critically involved in later steps of sperm activation, as one might expect functional redundancy with so many homologous proteins also present. An evolutionary context helps to clarify this observation; the large expansion of TTBKs is recent to *C. elegans* and absent from related *Caenorhabditis* species. Furthermore, the SPE-6 C-terminus is nearly identical among all members of the *Caenorhabditis* genus, but diverges extensively from the next closest paralog in *C. elegans* implying that other TTBKs have divergent functions. Outside of *Caenorhabditis*, sequenced genomes are primarily found in pathogenic nematodes; of these SPE-6 has higher BLAST matches in many

other Nematoda species than it does even in *C. elegans*, implying that the functionality of SPE-6 is highly conserved in Nematoda. Intriguingly, homologs of other genes required for sperm activation have not been found outside of *Caenorhabditis*, even though the basic aspects of MSP based sperm motility are conserved.

### **Central questions:**

The complete spermatogenesis arrest caused by null mutations of *spe-6* and the hyper abundance of *spe-6* alleles generated in *spe-27* suppressor screens point to a critical role for SPE-6 protein in *C. elegans* sperm function. We seek to understand the role of SPE-6 in *C. elegans* sperm and understand how it functions as a “molecular link” between the early step of Fibrous Body formation and the later step of sperm activation in sperm development. To do so, we used genetic, cytological, and biochemical methods to explore and contextualize SPE-6 function. The following specific questions were addressed in this thesis research:

-Where and when is SPE-6 protein expressed? Classical genetic studies suggest that SPE-6 is expressed within sperm themselves, but this assumption needs to be validated to understand SPE-6 function. A number of other proteins involved in FB-MO morphogenesis disappear before mature haploid spermatids are formed. Is SPE-6 like these, or does SPE-6 persist and have a functional role in spermatids or even later, in crawling spermatozoa?

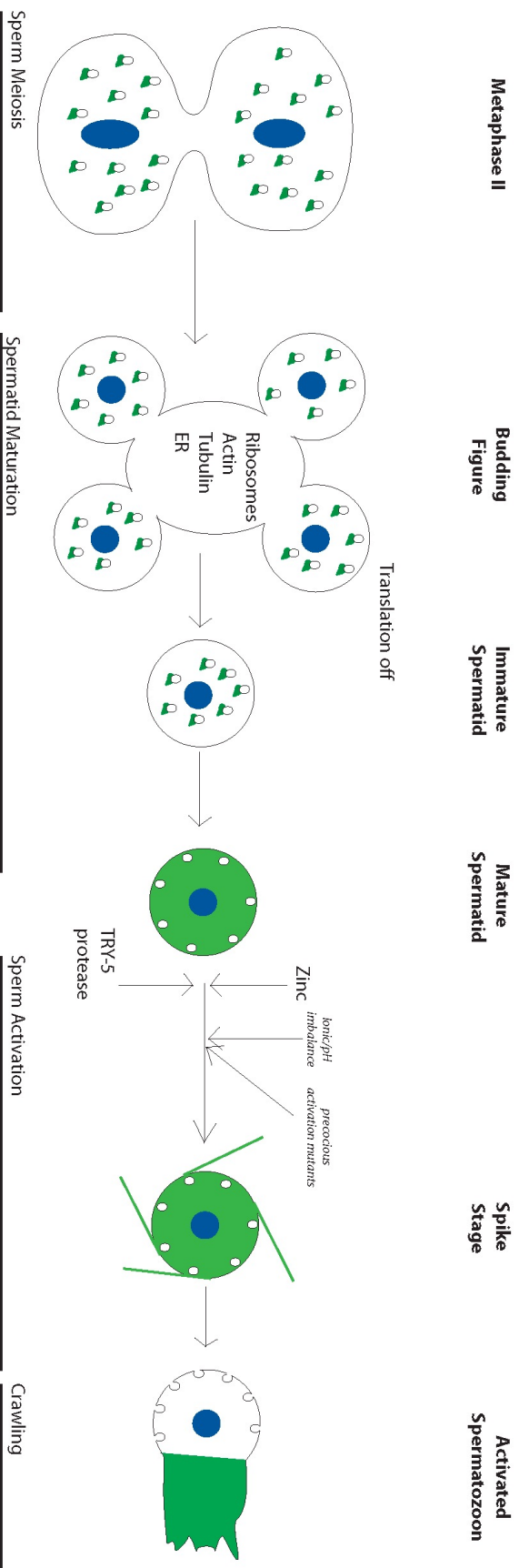


-Do *spe-6* “suppressor alleles,” missense mutations causing precocious activation in the absence of extracellular signals, cause a gain or loss of SPE-6 activity? Answering this question will help explain how SPE-6 regulates sperm activation and will also provide fundamental insight into the general strategy utilized by *C. elegans* sperm to regulate the important decision to activate.

-What are the genetic, cellular, and physiological requirements for SPE-6 function and localization? In other words, how does SPE-6 fit into what we already know about *C. elegans* spermatogenesis? A large classical genetic toolkit of well characterized sperm mutant alleles will allow sophisticated, hypothesis driven, exploration of these questions. Specifically, immunofluorescence analysis of SPE-6 in mutants defective for sperm activation can interrogate the genetic requirements for SPE-6 localization and determine whether SPE-6 is downstream of the SNF-10 pathway, the SPE-8 pathway, or both. Furthermore, MO fusion is an important event of sperm activation, but little is known about how this event fits into the regulatory pathway of sperm activation; analysis of SPE-6 in MO fusion mutants will allow further understanding

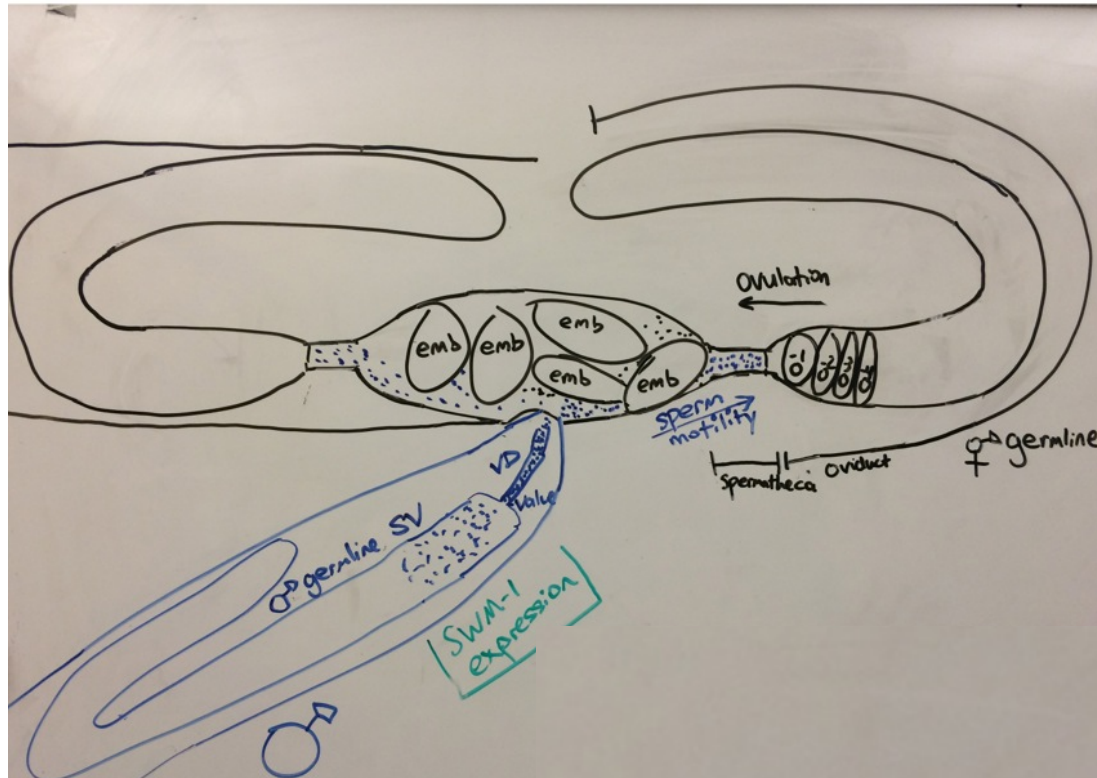
-How is SPE-6 itself biochemically regulated to accomplish its cellular function? Using a combination of biochemical methods and genetic methods, we can examine SPE-6 in different cell types and explore the question of whether SPE-6 is post-translationally modified.





**Figure I-1: FB-MO dynamics and sperm activation**

To progress through the meiotic divisions, the motility protein Major Sperm Protein (Green) is packaged within the Fibrous Body (FB) - Membranous Organelle (MO) (White) structures for successful meiotic divisions. This process requires SPE-6. Following the meiotic divisions, spermatids are formed from a polarized budding division ("Budding Figure"), where cytoplasmic "junk" including ribosomes, tubulin, and actin, are discarded in a central residual body. Following budding, spermatid maturation involves the breakdown of FBs into cytosolic MSP dimers, simultaneously the MOs dock at the cell membrane. Sperm activation begins in response to the extracellular signals TRY-5 (a secreted serine protease) or ionic zinc. In intermediate steps of sperm activation sperm, extend transient MSP containing spikes and MOs fuse with the cell membrane. Sperm acquire motility when MSP spikes coalesce into a single polarized pseudopod on one side of the cell. Notably, this entire process of sperm activation takes approximately only 10 minutes *in vitro* (Shakes and Ward, 1989). (For a review, see Chu and Shakes, 2012).



**Figure I-2: Reproductive anatomy of *C. elegans*.**

Blue: Male worm and male sperm. Black: Hermaphrodite. Looped structures are gonads, hermaphrodites contain two looped gonad arms whereas males contain one. Note on left the outlines of worm bodies- gonads are nearly half the mass of adult *C. elegans*.

Germline: site of germ cell (gamete) production

Oviduct: site of oocyte maturation (most proximal region of germ line)

SV: seminal vesicle storage for male sperm (most proximal region of germ line)

Spermatheca: valved structure where sperm "wait" for oocytes

Uterus: contains embryos (emb) before laying, sperm crawl through here before reaching spermatheca

Male sperm crawl through the hermaphrodite uterus until they reach the spermatheca. Since hermaphrodite sperm and oocytes are compartmentalized in the same area of the gonad, it might be expected that motility is dispensable for hermaphrodite sperm; in fact this is not the case. When oocytes move through the spermatheca into the uterus, they sweep sperm out of the spermatheca. Sperm must subsequently migrate back into the spermatheca to remain fertilization competent. Both hermaphrodite and male sperm are affected by the sweeping; however, male sperm (blue) consistently outcompete hermaphrodite sperm (black dots) for re-entry into the spermatheca and physically block hermaphrodite sperm entry.

**Figure I-3: SPE-6 Amino Acid sequence with annotated mutations.**

SPE-6 sequence of three members of the *Caenorhabditis* genus along with four other paralogous *C. elegans* Tau Tubulin Kinases. Note high conservation at the C-terminus specific to SPE-6 genes, but absent from outgroup paralogs.

C. bren SPE-6	1	--MSEPLRDSRGMPQPGFIIIEHQNSDHKKVLRNIYSGPFSDVYVVADTVTNEKYAM
C. rem SPE-6	1	-MSSEQLHDSRGMPQAGFIIIEHQSSDQKKVLRNIYSGPFSDVYVVADVATNEKYAM
C. ele SPE-6	1	--MSE-QRDSRGMPQAGFIIIEHQSSDHKKVLRNIYSGPFSDVYV ADTVTNEKYAM
C09B9.4	1	MVASPHRNSINDLLMPLLGKRIRLGDHVYKMCDSIATGPFSSVFLVEK--DGIPYAM
C39H7.1 MPAK	1	-----MAQELIQINPGQQVERWSIEKKLGEGGFGAVYRVFDTAG---KYA
F36H12.8 TTBK-1	1	-----MTAPPPPLVELPPGSMVERWSITKKLGEGGCGAVYLCCTDATG---KYA
R90.1 TTBK1/2	1	-----MTSSSEGEILQVGQVIRERWKIKAKIGGGGFGGEIYEATDVQNHHERVA

\*hc163 V to E

C. bren SPE-6	56	KCERQEGNSRPVLKLDVMVLMATK--GLKGFPNFVAAGRTEVYRYCIMQLVGPD LGR
C. rem SPE-6	57	KCERQEGNTRPVLKLDVILVLMATK--GLKGFPKFVAAGR TDVYRYCIMQLVGPD LGR
C. ele SPE-6	55	KCERQEGNSRPVLKLDV MVL MATK--GLRGFPNFVAAGR TDVYRYCIMQLVGPD LGR
C09B9.4	56	KVESQSKCLRPVLKLDHAVLRALG--HQSGFPSLTSAGR TENFKYVVMQLVGPD LSM
C39H7.1 MPAK	43	MKVEGANEQIQVLKLEVSVLNELSKRGNRHFKIEDKGRFGNFNYVVM TLVGKSLQD
F36H12.8 TTBK-1	46	LKVEGISEAMQVLKMEVLVLGELTKRGSRHFKIEDKGRYGSFNYVVM TLVGKSLQD
R90.1 TTBK1/2	49	IKVESSKATKQVLKMEVAVLRRLQ--GKKHACKFYGCGRNDKFNYLVMSLQGNLAD

\*hc187 G to E

C. bren SPE-6	111	LRRTRPERRFSLPTALQILGQTLRRLEDLHNC GWLCRDVKAPNFCIGIGENEST--
C. rem SPE-6	112	LRRTRPERRFSLPTALQILGQTLRRLEDLHKCGWLCRDVKAPNFCIGIGEH EST--
C. ele SPE-6	110	LRRTRPERRFSLPTALQILGQTLRRLEDLHNC GWLCRDVKAPNFCIGVGENEST--
C09B9.4	111	LLEFAPQQRFTSSITVYKIALQTLDRVLHEAGWLN RDVKAQNFVAVGLGEESI--
C38H7.1 MPAK	100	LNKAGVGGHMSMGCSIGIGIQSLEALEDLHNIGYLHRDVKPGNYTIGRPELNEIRK
F36H12.8 TTBK-1	103	LRKGTAAQQCLSLACSLSVGIQSLEALEDLHNIGYLHRDVKPGNYTIGRAELNELRK
R90.1 TTBK1/2	104	LRREAPKQCENLSTAVRVGIQILNGIREIHSIGFLHRDVKPSNFAMGR-TSQ TMRN

\*hc92 T to I

C. bren SPE-6	165	VYILDFGFARKFVDKEGKIIPPRSAAALMGTFQYCAVSAHSHKDQC RDDLESWFY
C. rem SPE-6	166	VYILDFGFARKFIDKEGKIIPPRSAAALMGTFQYCAVSAHSHKDQC RDDLESWFY
C. ele SPE-6	164	VYILDFGFARKFVDKEGKIIPPR TAAALMGTFQYCAVSAHSHKDQCARDDES WFY
C09B9.4	165	VYMLDFGLTRKYLEHNGSRSLRPHGPSVGTFFPYAPLASLGFC DQSPIDDI EGWLY
C38H7.1 MPAK	156	VYILDFGMCRKFTGNNGTIRKPRQAAGFRGTVKYAPI SCHLQRELCKRDDLETW MY
F36H12.8 TTBK-1	159	VYILDFGMARKEFTDNGGVIRKPRAAAGFRGTVRYAPIACHKNQELGRKDDVE VWLY
R90.1 TTBK1/2	159	VYMLDFGLARQYINAKGEIRSPRSAAGFRGTVRYAAVTAHKNKEMGRQDDLW SLY

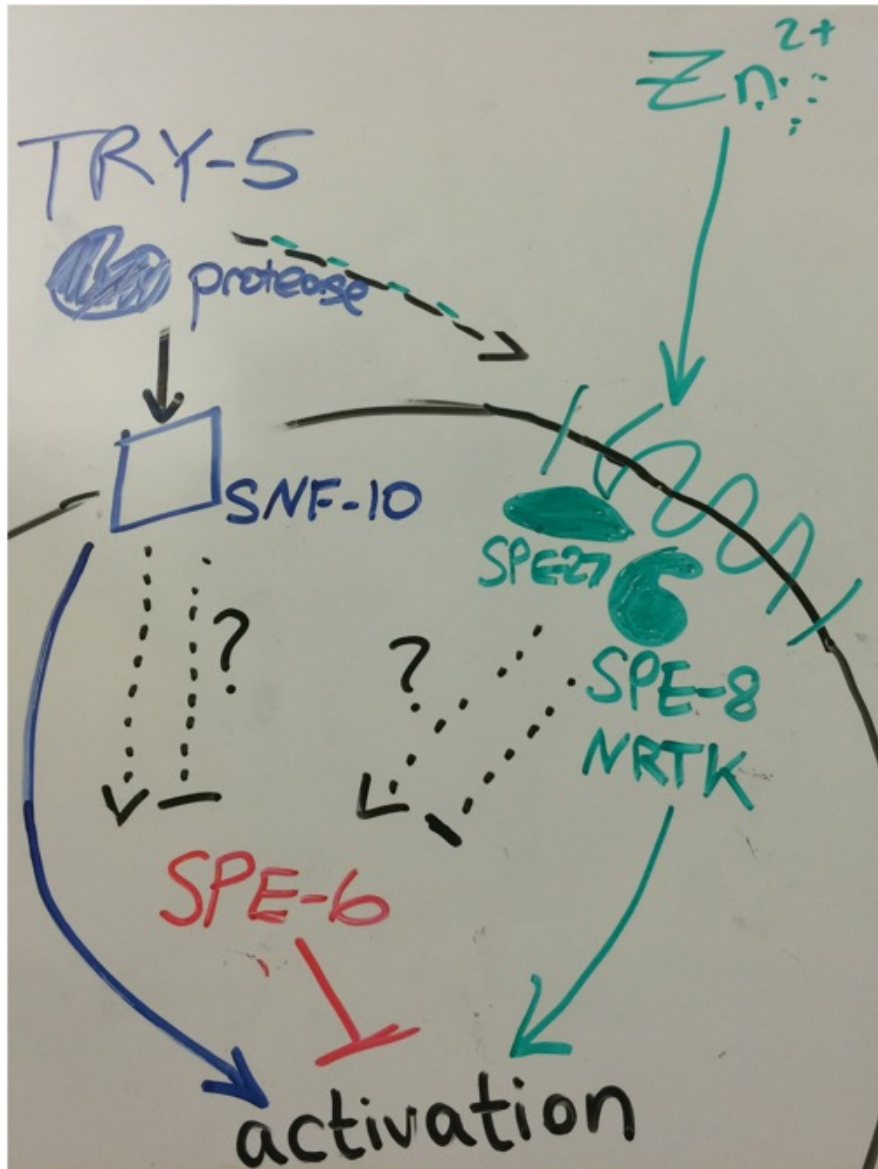
\*hc49 Q to stop

C. bren SPE-6	221	MAVELLKGPLPWANIDGHKNHKLIGDAKMEIRNEPARSEFFEGVPRQFNEILTILD
C. rem SPE-6	222	MAIELLKGPLPWANIDGHKNHKLIGDAKMEMRTEPARSEFFEGVPKQFNEILSILD
C. ele SPE-6	220	MGIELLKGPLPWANIDGHKNHKLIGDAKVAIRSEPLRSEFFEGVPKQFNEILTILD
C09B9.4	221	MTVHLLKGGPLPWHNSKRALNLPKVREWKMYCRRPGGKHYLEFAGIPKGWADIFDVIV
C38H7.1 MPAK	212	MOVELSHGTIPWQHIS---DMNQVGQAKQAIRNNLGLTFPPPCPQ-QFQDIMRMVD
F36H12.8 TTBK-1	215	MOVELTVGRVPWKEIT---DMNAVGOAKQAIRNTPEKMFVFPCPANELKEIMKMVD
R90.1 TTBK1/2	215	MLTEFLQGQLPWRKIK---DKDEVGKMKEEAD---LVVLLDDCPH-EMHLFVAHLK

C. bren SPE-6	277	ATSEFDRPNYTKLGELLAEAAAEHKVTLKEPLDWQNNERMQQKAIFVG---ELGES
C. rem SPE-6	278	STSEFDRPDYKRLSELLREAAAEHQVTMKEPLDWQNNERMQQKAIFVG---ELGES
C. ele SPE-6	276	QTSYFDRPNYKKLGDLLSQAATEHQVTLKEPLDWQNNERMQQKAIFVG---ELGES
C09B9.4	277	NTAPHETPDYNKIANMVLSIARNELIDL TAPFDWQVN-PVLRSLVRLG---PLSCG
C38H7.1 MPAK	264	AMKYADAPNYQAIYGMRRQAYGACGSNENAPYDWENG GP-----AAYL
F36H12.8 TTBK-1	268	SWDYFADPNYADCYRLMKQTLANCGKPE-YPYDWE PGMP-----LNYM
R90.1 TTBK1/2	264	VLGYADTPDYVYLESLLNKIVAENEISWDEPYDWELGYDNMATRQKQQANGNLSAR

C. bren SPE-6	330	HEASAKLDAKDNPNESMDIEFDDHP-KETLSKSISAEKACACAWFISTCLAQT [+334]
C. rem SPE-6	331	HQASAKLDAKDNPNESMDIEFDEIP-----
C. ele SPE-6	329	HQASAKLDAKDNANESMDIEFDDMPPKEGISKSLSAEKSCTKN--VETARTEK---
C09B9.4	329	ELTRIPMSSMAATPFIDQILLNSIGSPLESY-----
C38H7.1 MPAK	307	LH-----
F36H12.8 TTBK-1	310	VK-----
R90.1 TTBK1/2	320	LKSHTTAVIKDQRNENRALDTQAPITMGEDEDEQTNGQMANLGAGFESTHEKA [+403]

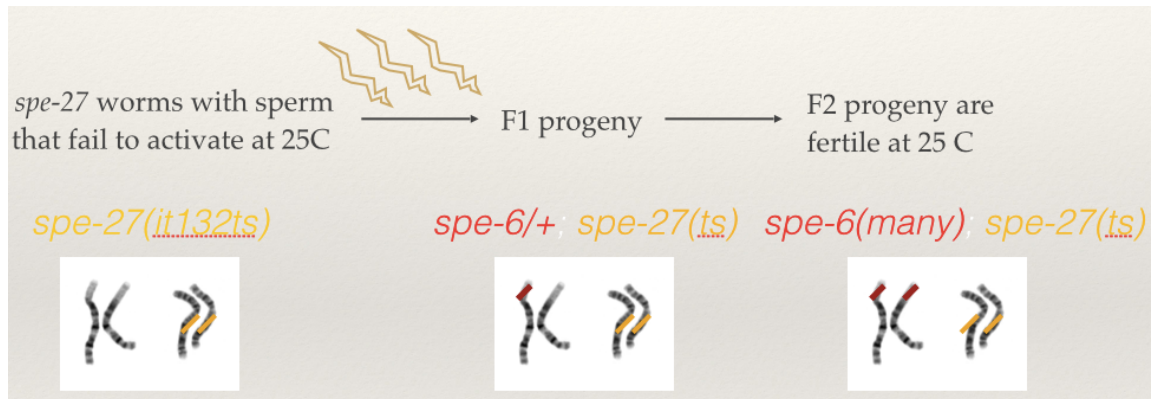
predicted substrate binding



**Figure I-4: Cartoon of extracellular and membrane bound sperm activation pathway components.**

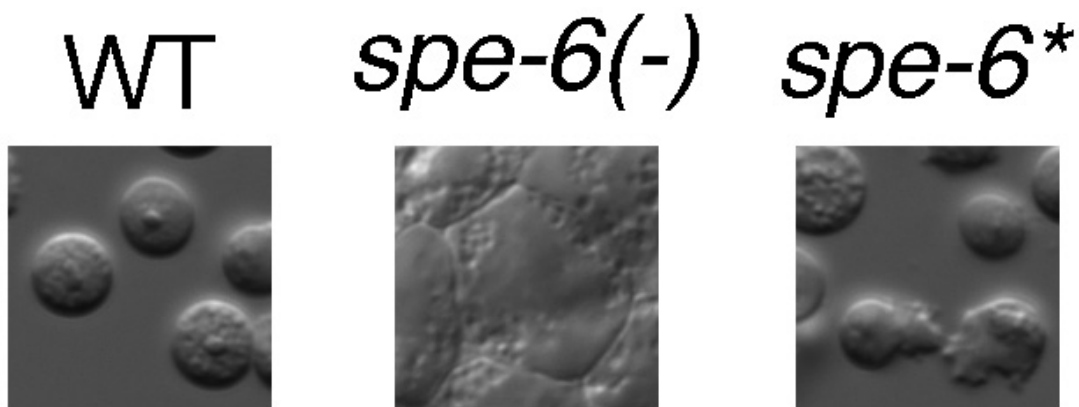
Arrows indicate genetic rather than physical interaction. "NRTK" stands for Non-Receptor Tyrosine Kinase, the identity of the *spe-8* protein product.





**Figure I-5: Identification of *spe-6* suppressors of *spe-27* (*spe-8* class).**

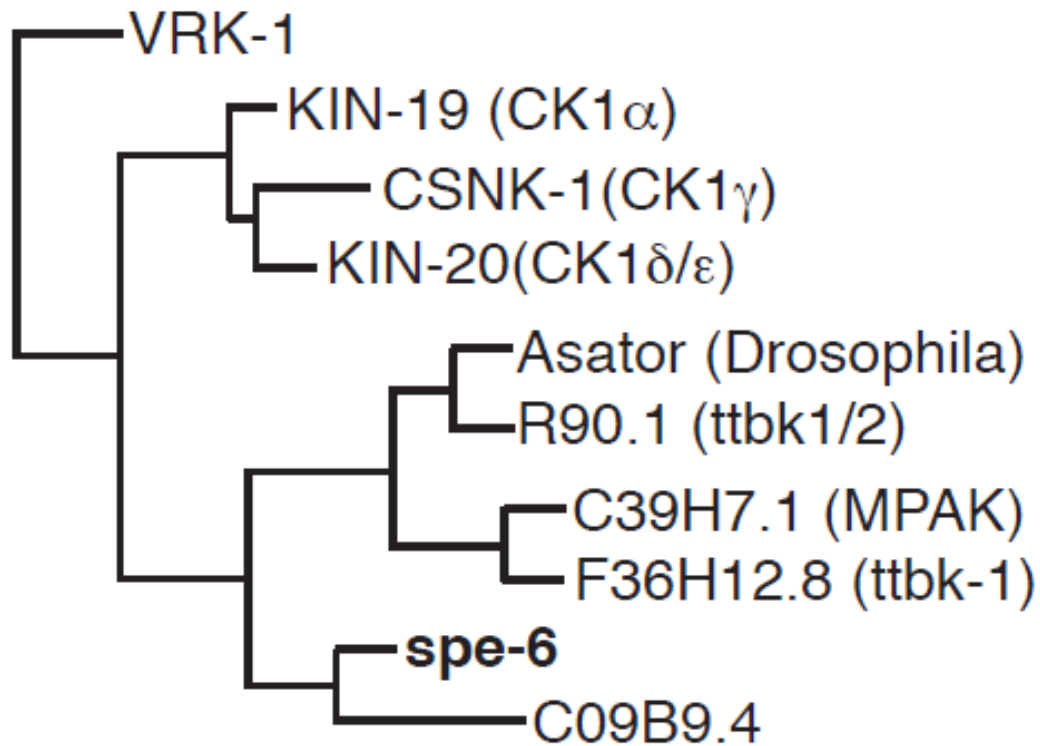
Forward genetic suppression screens identified a number of alleles of *spe-6*. The combination of these mutated alleles with *spe27(it132)* (a temperature sensitive *spe-8* class mutant) restored fertility and the capability for sperm to activate. Analysis of the *spe-6* alleles alone showed that *spe-6\** special activation alleles caused precocious activation in the absence of extracellular signals. (Muhlrad and Ward 2002).



**Figure I-6: Wildtype, *spe-6(-)* (null), and *spe-6\** terminal phenotypes in the male Seminal Vesicle.**

DIC micrograph of inactivated spermatids (WT), arrested spermatocytes (*spe-6(-)*), and precociously activated spermatozoa (*spe-6\**). SPE-6 is absolutely required for early spermatogenesis and FB-MO formation, but its second functional role in sperm activation is not well characterized - *spe-6\** alleles have unpredictable effects on





**Figure I-7 SPE-6 phylogenetic context:**

SPE-6 is a divergent member of the Tau Tubulin Kinase sub-group of Casein Kinase 1s. TTBK-2 is the *C. elegans* kinase with highest sequence homology to human Tau Tubulin Kinase. R90.1 is the best match to ttbk1/2 in *C. elegans*. Tree was generated using NCBI protein blast and Clustal W.

## **Results:**

### **Construction of a polyclonal antibody specific to SPE-6:**

To better understand how SPE-6 may regulate sperm function, we constructed a polyclonal antibody specific to an optimal peptide sequence from the protein's C-terminus (Figure 1A; see materials and methods). Our antibody bound a protein at the correct molecular weight of 42KDa in wild type males, but failed to bind in either *fem-3(e2006)* hermaphrodites which produce no sperm or in *spe-6(hc49)* males whose truncated SPE-6 product lacks the antigenic site (Varkey et al. 1993; Hodgkin 1986) (Figure 1B). In addition, indirect immunofluorescence analysis of worm gonads found that the peptide antibody recognized a protein in sperm cells in wild type males and in *spe-6(hc49)* recombinant heterozygotes, but not in *spe-6(hc49)* homozygotes (Figure 1C). Furthermore, our antibody bound sperm fated cells in wild type hermaphrodites with an identical pattern to that in males, but we failed to detect signal in oocyte fated tissue (Figure 1D). Therefore our antibody specifically detects SPE-6 protein in male and hermaphrodite sperm.

### **SPE-6 protein localization in wildtype spermatogenesis:**

To examine the sub-cellular patterns of SPE-6 localization and how this pattern might change in during the progression of spermatogenesis, we examined the localization pattern of SPE-6 in both intact wildtype male gonads

and those that had been flattened to generate a cellular monolayer. Expression of SPE-6 protein is absent from non-differentiated germ cells and instead begins expression in pachytene of sperm meiosis, consistent with the turn on of a number of other sperm genes (Chu and Shakes 2012) (Figure 2A). Cytological analysis of released spermatocytes determined that SPE-6 localization changes at multiple key transitions in spermatogenesis, shifting from a “granular” plus diffuse cytoplasmic pattern in spermatocytes to a perinuclear pattern in spermatids (Figure 2B). The granular plus cytoplasmic pattern in spermatocytes persists until during anaphase II and the early budding division, SPE-6 granular structures clearly localize to the budding spermatids while the diffusely localized SPE-6 concentrates in the residual body (Figure 2B and 2C). SPE-6 begins shifting to a perinuclear localization pattern during the course of the budding division, and is entirely localized perinuclearly in most, but not all, spermatids (Figure 2C). *C. elegans* sperm fail to reform a nuclear envelope, and instead the chromatin is encased in an electron rich material staining positively for RNA (Wolf et al., 1977); this structure termed the perinuclear halo is the likely location of SPE-6 in spermatids. In spermatozoa from wild type mated or *swm-1(me87)* males, SPE-6 displays a variable phenotype with SPE-6 localizing to the pseudopod, but often with some protein remaining perinuclear (see below).

**SPE-6 fails to localize to FB-MOs, is insufficient for fibrous body formation, but does leave the cytosol coincident with FB breakdown:**

Since SPE-6 is required for assembly of the major sperm protein (MSP)

into fibrous bodies (FBs), we questioned whether the granular pattern of SPE-6 staining might correspond to either FBs or the associated membranous organelles (MOs). To test for FB localization, spermatocytes and budding figures were co-labelled with antibodies against SPE-6 and MSP. In such preparations, SPE-6 and MSP failed to co-localize, although clear adjacencies were common (Figure 3A). To test whether SPE-6 was localizing to the associated membranous organelles (MO), spermatocytes were co-labeled with antibodies against SPE-6 and an MO marker (the monoclonal antibody 1CB4). However, these two patterns also did not overlap (Figure 3B). Together, these results suggest that SPE-6 may preferentially localize to some, as yet unidentified cytoplasmic granules that are often near but are distinct from FB-MOs. While this localization pattern may still be important to SPE-6 function in promoting FB formation, an additional hypothesis is that SPE-6 localization to cytoplasmic granules prevents disposal of SPE-6 in the residual body and functions as a vehicle for SPE-6 delivery to spermatids (See below).

We further questioned whether SPE-6 expression might coincide with fibrous body formation. MSP is expressed in the cytosol during the pachytene stage of meiosis, coincident with a number of other sperm genes. Yet, MSP does not polymerize into FB-MOs until late pachytene. If expression of SPE-6 is sufficient to promote MSP assembly into FB, we hypothesized that the initial expression of SPE-6 would coincide with early FB formation. However when we co-labeled male gonads with antibodies against MSP and SPE-6, SPE-6 and MSP exhibited perfectly overlapping expression patterns, with the two proteins

turning on in the same cell of the germ line (White arrow, Figure 3C). This coincidental-expression of SPE-6 and MSP prior to FB-MO formation indicates that SPE-6 protein expression alone is not sufficient to induce FB-MO formation.

In monolayer preparations of spermatids, most spermatids were found to have SPE-6 exclusively localized to the perinuclear halo. However, these preparations always included a few spermatids in which SPE-6 was both perinuclear and cytosolic, a pattern that was more typical of budding spermatids (Figure 2C). To determine whether these were newly detached, immature spermatids, sperm monolayers were co-labeled with anti-MSP and anti-SPE-6 antibodies. During the process of spermatid maturation, FBs detach from the MOs and the FBs breakdown as MSP de-polymerizes into cytosolic dimers. Analysis of double-labeled spermatids revealed that SPE-6 is only present in the cytosol in immature spermatids that have not yet disassembled their FBs (asterisk labelled spermatid in Figure 3D); in mature spermatids, SPE-6 fully localizes to the perinuclear halo (Figure 3D). Therefore, localization of SPE-6, exclusively to the perinuclear halo coincides with spermatid maturation and FB breakdown.

**The *spe-6(hc163)* lesion causes SPE-6 protein mislocalization and abnormal segregation to the residual body:**

The *hc163* mutation encodes a valine to glutamic acid lesion at a conserved residue in the N-terminal ATPase domain (Figure 1:). The robust suppression of the *spe-8* class phenotype exhibited by *hc163* led to this allele

being selected for subsequent genetic and cytological study (Muhlrad and Ward, 2002). To further understand how this specific mutation leads to precocious activation, we examined SPE-6 protein localization using our peptide antibody. *hc163* SPE-6 appears to turn on normally and be present at normal levels in spermatocytes (Figure 4A). Strikingly, SPE-6 levels are reduced in spermatids and absent from perinuclear halos in both spermatids and spermatozoa (Figure 4A). Because reduced levels of mutant SPE-6 in spermatids could arise from either mislocalization during the budding division or from proteolytic degradation, we analyzed SPE-6 patterns in sperm monolayers. In meiotically dividing mutant spermatocytes, SPE-6(*hc163*) is primarily cytosolic rather than present in discrete cytoplasmic granules, a phenotype that is particularly noticeable when comparing wildtype and *hc163* budding figures (Figure 4B). Furthermore, during the budding division that follows anaphase II, most of the *hc163* form of SPE-6 protein segregates to the residual body rather than to the spermatids, thus accounting for the lower levels in mutant spermatids (Figure 4B). Despite the aberrant localization, western blots detected no mobility shift of the mutant SPE-6 product implying that full length rather than truncated protein is being mislocalized (Data not shown and examples below, figure 5C). Therefore, the reduced levels of SPE-6 in *spe-6(hc163)* spermatids stems from the improper segregation of *hc163* SPE-6 into the residual body. This mis-segregation to the residual body may, in turn, be linked to an earlier alteration in the localization of SPE-6 within developing *hc163* primary spermatocytes.

Although SPE-6 protein levels appear reduced in *hc163* spermatids, the neomorphic localization pattern complicated our analysis of how the precocious activation phenotype might develop. Based on these initial observations, we considered two competing models regarding the function of wild type SPE-6 and how mutant alleles may cause precocious activation. Consistent with previous proposals (Muhlrad and Ward, 2002), the first model assumes that SPE-6 functions as an inhibitor of sperm activation; thus the *hc163* phenotype stems from lower protein levels in the spermatids due to the missegregation of SPE-6 away from the spermatids and into the residual body. However our results are also consistent with a second model in which SPE-6 functions as a positive regulator of sperm activation. In this second model, non-sequestered, cytoplasmic *hc163* SPE-6 acts catalytically within spermatids to stimulate precocious sperm activation.

***spe-6(hc163)* protein localizes independently of wild type protein, but is recessive for male precocious sperm activation:**

Gain of function mutations as suggested by this second model are typically, but not always, genetically dominant. Although previous studies had shown that the ability of *hc163* to suppress the self-fertility of *spe-27* hermaphrodites was genetically recessive, these studies did not demonstrate how *hc163* might be functioning in the context of male sperm activation. If mislocalized or otherwise over-active *hc163* protein is functioning in a gain of function manner within male spermatids, we hypothesized that it might cause

precocious activation in males. However, *spe-6(hc163)* heterozygote males failed to exhibit precocious activation in our analysis, with only 4 out of 45 gonads containing any activated spermatozoa as compared to 100% (52/52) of *hc163* homozygotes (figure 4C). Therefore, *hc163* is recessive for sperm activation in males as well as hermaphrodites. To rule out the possibility that in *hc163* heterozygotes the wildtype protein was interacting with the *hc163* protein and “correcting” its localization defect, we examined the localization of SPE-6 in the spermatids of *hc163/+* heterozygotes (Figure 4D). In such spermatids, SPE-6 was present both in the perinuclear halo and in the cytoplasm, consistent with the two allelic forms of the protein each localizing independently of the other. Taken together these results suggest that *hc163* is a neomorphic mutation that results in altered protein localization, yet is recessive at the phenotypic level. If *spe-6(hc163)* encodes a gain of function mutation, the amounts present in the cytoplasm of *hc163/+* spermatids are insufficient to induce precocious sperm activation.

***hc187* is a temperature sensitive *spe-6* hypomorphic allele at both the protein and phenotypic levels:**

Although the *hc163* mutation is genetically recessive at the phenotypic level, its neomorphic protein localization precludes the unambiguous designation of *hc163* as a classic loss of function allele. We therefore chose to analyze a second mutant allele that had been recovered in the *spe-27(it132)* suppressor screen, *hc187*. The specific alteration in *hc187*, a G to E substitution in a



conserved in Tau Tubulin Kinase residue, was found in two independently isolated *spe-6* suppressor-class alleles, suggesting that this residue is functionally significant (Figure 1A; Muhlrads and Ward, 2002). Phenotypic analysis of celibate males of the single mutant revealed that, like in *hc163*, *spe-6(hc187)* single mutant males exhibited precocious sperm activation, suggesting that *spe-6(hc187)* also generally bypasses the normal requirements for sperm activation signaling (See below).

To further understand how this amino acid substitution was affecting the function of SPE-6, we analyzed the localization pattern of SPE-6 in *hc187* mutants. In our initial studies, the localization patterns of SPE-6 appeared generally normal, however we observed a surprisingly degree of experiment to experiment variability in the relative protein levels. Since the *spe-27(it132)* suppressor screen was conducted at a non-permissive temperature, we hypothesized that *spe-6(hc187)* might be temperature sensitive for either function or stability. We therefore analyzed *hc187* protein levels in males raised either at 16°C (generally permissive) or 25°C (generally restrictive). Strikingly, *hc187* SPE-6 exhibited near wild type levels of protein expression at the permissive temperature, but drastically lowered levels at the restrictive temperature (Figure 5A and 5B). Importantly, no such temperature sensitivity in SPE-6 protein levels was detected in wild type, *swm-1(me87)* controls, or in *spe-6(hc163)* (Figure 5B). Therefore, *spe-6(hc187)* encodes a temperature sensitive loss of function mutation resulting in dramatically lowered protein levels at the non-permissive temperature.

The unambiguous temperature sensitive loss of function effect in *hc187* allowed an excellent opportunity to test whether lower protein levels of SPE-6 are sufficient to induce precocious activation. We examined gonads from celibate males raised at the permissive vs. non-permissive temperature with immunofluorescence and scored for precocious activation as above. We observed a striking difference in precocious activation between the two temperatures; less than five percent of *hc187* worms at the permissive temperature, but all *hc187* worms analyzed at the restrictive temperature, exhibited precocious activation (Figure 5C and 5D). Parallel analysis in *him-8*, *swm-1*, and *spe-6(hc163)* backgrounds found no sensitivity of precocious activation to temperature, confirming that the temperature sensitive phenotype is specific to *spe-6(hc187)*. Based on these data linking *hc187* temperature sensitive protein expression to precocious activation, we conclude that lowered SPE-6 expression is sufficient to induce precocious activation. Therefore, the *spe-6\** mutations causing precocious activation are hypomorphic alleles, supporting the hypothesis that, in wild type, SPE-6 may function as a “brake” to sperm activation.

We next questioned what changes in SPE-6 protein correspond to the temperature sensitive precocious activation phenotype in *spe-6(hc187)*; for instance we might predict that SPE-6 levels are uniform at lower temperatures until a certain threshold temperature when SPE-6 protein loses ability to fold inducing precocious activation. Further analysis of *spe-6(hc187)* with both DIC and anti-MSP immunofluorescence determined that precocious activation is first

induced at a temperature between 20 and 22° C (Figure 6A and 6C). Strikingly, robust differences in precocious activation are observed between these two temperatures, with no evidence for an intermediate precocious activation phenotype at intermediate temperatures. Although we have not quantitatively evaluated SPE-6 levels at intermediate temperatures, we do observe a trend of decreased protein expression as temperature rises (compare SPE-6 levels at 22°C and 25°C in Figure 6C). We questioned whether temperature might modulate the capability of *spe-6(hc187)* to suppress *spe-8* class sperm activation defects. Indeed, *spe-6(hc187)* suppressed the infertility of *spe-29(it127)* in a temperature sensitive manner, with increasing broods at higher temperatures (Figure 6B). Notably, *hc187* also partially suppressed the self-sterility of *spe-29(it127)* hermaphrodites at 20°C, a temperature at which we failed to see high levels of precociously activated sperm in the seminal vesicle (no precociously activated sperm observed in 17/22 male gonads). Thus 20°C may be a threshold temperature at which SPE-6 levels are sufficiently low to allow bypass a loss of *spe-29* in of hermaphrodite sperm but insufficient to stimulate precocious activation of male sperm within the seminal vesicle

We also undertook temperature upshift experiments with *hc187* animals raised at a permissive 16° C. Some temperature sensitive mutations primarily affect protein synthesis and initial folding while others can disrupt the function of already synthesized protein. After 3 hours upshifted to a restrictive temperature, we observed displacement of mutant SPE-6 from the perinuclear halo in some but not all spermatids, yet this effect failed to elicit widespread precocious

activation (figure 6B and 6D). Therefore, while *spe-6(hc187)* is not a fast acting temperature sensitive mutation, mutant protein conformation may still be sensitive to increased temperature following protein synthesis and result in altered protein localization.

### **SPE-6 is dispensable for sperm motility:**

The localization of SPE-6 to the pseudopod in spermatozoa led us to question whether SPE-6 might function in regulating motility. SPE-6 is homologous to the *Ascaris suum* kinase, MPAK, which regulates MSP polymerization dynamics at the leading edge of the pseudopod (Yi et al. 2007). Both MPAK and SPE-6 are strongly implicated in the polymerization of MSP; SPE-6 in fibrous bodies and MPAK in the pseudopod of motile sperm (Varkey et al., 1993). However, while our phylogenetic sequence analysis shows that both SPE-6 and MPAK are broadly homologous to Tau Tubulin Kinases, neither is *spe-6* the closest MPAK homolog in *C. elegans* nor is MPAK the closest *spe-6* homolog in *A. suum* (Figure 7A). To further address a possible role of SPE-6 in regulating motility in already crawling spermatozoa, we compared the migration of precociously activated *swm-1* spermatozoa to those of *spe-6(hc187)* at 25°C, a condition where SPE-6 protein levels are highly depleted. Not only were *hc187* spermatozoa fully motile, their average velocity exceeded that of *swm-1* spermatozoa ( $p = .008$ ) (Figure 7B and 7C). Therefore, partial depletion of SPE-6 levels does not reduce sperm motility, suggesting that SPE-6 is not

required for pseudopod treadmilling. In contrast, this evidence may implicate SPE-6 as a negative regulator of sperm motility.

**SPE-6 may be absent from the perinuclear halo in non-motile spermatozoa:**

Given the cytological and genetic evidence for SPE-6 involvement in sperm activation, we sought to further characterize the SPE-6 localization change during this process (enhanced versions of all patterns described here found in Figure 8E). *swm-1(me87)* male spermatozoa precociously activated within the male seminal vesicle consistently contained SPE-6 either within both the pseudopod and perinuclear halo (Figure 8A, blue arrows) or exclusively within the pseudopod (Figure 8A, white arrows).

Because sperm are produced continuously over time, one possible explanation of the observed variability of *swm-1* sperm is that they represent a mix of sperm at various stages in an extended activation process and/or in a post-activation state of decline. Furthermore, spermatozoa with SPE-6 in both the pseudopod and the perinuclear halo might represent an intermediate state as SPE-6 moves from the perinuclear halo to the pseudopod. To study a synchronously activated population of sperm, we activated spermatids *in vitro* using pronase and then processed samples at noted time points for anti-SPE-6 and anti-MSP immunofluorescence. To insure that we were including intermediates in our analysis, we included samples in the spike stage, a known intermediate in the activation process (Shakes and Ward, 1989). During this stage, thin, MSP-containing spike or filopodia-like structures extend from all sides

of the cell before coalescing into a single polarized pseudopod. We found that sperm spikes occasionally survived fixation and staining protocols and were detectable with MSP staining (Figure 8B green arrowhead). In these cells, SPE-6 was restricted to the perinuclear halo in a pattern that was indistinguishable from that of normal, unactivated spermatids. In early time points, we also identified spermatozoa that had not extended full pseudopods (figure 8A “short”). In these “short” pseudopod sperm, the MSP was fully polarized but only a small amount of SPE-6 overlapped with the MSP signal. To our surprise, spermatozoa that had been fully activated with pronase (Figure 8B “zoa”) always had SPE-6 present in both the pseudopod and the perinuclear halo. Under DIC optics, spermatozoa at these time points were motile and are considered to be fully activated spermatozoa (Shakes and Ward 1989 and data not shown). Therefore, although SPE-6 is variably localized in *swm-1* precociously activated spermatozoa, SPE-6 is invariably localized to both the pseudopod and the perinuclear halo in *in vitro* pronase activated spermatozoa. Because *swm-1* animals contain some activated but non-motile spermatozoa (data not shown), we hypothesize that SPE-6 in both the perinuclear halo and the pseudopod is a feature of actively treadmilling spermatozoa whereas SPE-6 entirely localized to the pseudopod is a feature of non-motile spermatozoa.

To further understand how SPE-6 might be functioning in activated spermatozoa, we examined SPE-6 localization in spermatozoa that had been activated *in vivo* within the hermaphrodite reproductive tract. Feminized *fog-2(q71)* “females” fail to make their own sperm; therefore the only sperm

within mated *fog-2(q71)* animals are those contributed by males. We allowed wild type males to mate with *fog-2* “females” for 24 hours and then processed the mated “females” for immunofluorescence. Surprisingly, we consistently saw that SPE-6 localization was dependent on the location of sperm within the hermaphrodite reproductive tract. In sperm within the uterus, SPE-6 was typically present in both the pseudopods and the cell body. While some uterine sperm contained SPE-6 in both the pseudopod and perinuclear halo, a unique feature of most of these uterine-localized sperm was the localization of SPE-6 to puncta throughout the cell body (Figure 8D “uterus”). In contrast, SPE-6 localized entirely to the pseudopod of spermatozoa within the spermatheca, although occasionally we would find atypical spermathecae whose sperm had SPE-6 present in both the cell body and the pseudopod (Figure 8D “normal” and “rare”). Given the SPE-6 pattern in uterine spermatozoa, we suspect that sperm within the spermathecae that had SPE-6 in both the pseudopod and the cell body might be motile spermatozoa returning after a recent ovulation event. The context dependent localization of SPE-6 in spermatozoa came to our great surprise as no study has described molecular differences between spermatozoa depending on the spatial location of the sperm themselves (SPE-6 patterns compared side by side in 8E). The finding that SPE-6 localization patterns are dynamic within activated spermatozoa implies that SPE-6 may perform regulatory functions within crawling spermatozoa beyond its initial function in blocking precocious sperm activation.

There are two features of sperm within the spermathecae that may be relevant to these observations. First, migration of sperm beyond the spermathecae and into the oviduct is regulated by some yet unknown mechanism that could be repressing the motility of sperm within the spermathecae (Ting et al. 2014). Second, the spermathecae is a highly elastic tissue that undergoes significant expansion and retraction as unfertilized oocyte enter and fertilized oocytes exit. Physical and/or signaling interactions between the spermatozoa and the somatic cells of the spermathecae could differ significantly between an oocyte occupied and oocyte unoccupied state and both are likely to differ from the microenvironment of the uterus. Taken together, the differential localization of SPE-6 in *in vivo* activated spermatozoa are consistent with a model where SPE-6 localization in spermatozoa correlates with the location of the sperm within the reproductive tract of the hermaphrodite and to whether or not the activated spermatozoa are fully motile.

**SPE-6 localization in activated spermatozoa is not absolutely dependent on either sperm activation pathway or MO fusion:**

In double-mutant experiments, *spe-6\** alleles have been found to bypass loss-of-function mutations in both arms of the activation response pathways; more specifically, male sperm activate precociously in double mutants combinations with both mutants (Figure 9A). To further explore these relationships, we questioned whether mutations either in *spe-8* class genes or in *snf-10* would alter the pattern of SPE-6 localization in activated spermatozoa. To



address this question, we first activated mutant spermatids with the *in vitro* activator pronase and processed slides for immunofluorescence as above. Consistent with previous reports, spermatids of the *spe-8* class mutant *spe-27(it110)* failed to activate in pronase, but extended MSP-containing spikes, implying that an intermediate stage of sperm activation is achieved (Shakes and Ward, 1989). Despite this intermediate stage of sperm activation, SPE-6 failed to leave the perinuclear halo and was indistinguishable from wild type spermatids (Figure 9B). We next analyzed SPE-6 localization in *snf-10(hc193)* mutant sperm, which fail to undergo any morphological changes in response to pronase (Fenker et al., 2014). In preliminary experiments, some SPE-6 protein appeared cytosolic after *snf-10(hc193)* sperm were exposed to pronase. Therefore, the *spe-8* pathway, but possibly not *snf-10*, is required for SPE-6 to leave the perinuclear halo when sperm are exposed to pronase. One interpretation of this result is that some cellular signaling upstream of SPE-6 may be elicited in pronase activated *snf-10(hc197)*, but that this signaling is absolutely dependent on the *spe-8* pathway. These results raise the question of whether the *spe-8* class is absolutely required for SPE-6 localization.

Although both *snf-10* and *spe-8* class male spermatids are insensitive to pronase, redundancy in the sperm activation pathway allows these same sperm to activate when males mate with hermaphrodites (Figure 9A). To test whether SPE-6 localization to the pseudopod requires either *spe-8* or *snf-10*, we mated males deficient for these genes to a feminized *fog-2(q71)* “female”. Importantly, activation via these two pathways could be location dependent within the

reproductive tract of the hermaphrodite; activation via the *spe-8* pathway should predominate within the spermatheca due to the local release of zinc (Liu et al., 2013) whereas activation via SNF-10 should predominate within the uterus near the site of insemination where TRY-5 levels are expected to be the highest (Smith and Stanfield, 2012). In this model, *spe-8* sperm should exclusively exhibit the “double pattern” and *snf-10* sperm should exclusively exhibit the pseudopod only pattern. Analysis of these naturally activated *snf-10* and *spe-8* spermatozoa revealed that SPE-6 localized in a location dependent manner that was independent of the *snf-10* or *spe-8* mutations (Figure 9C). As we had observed in wildtype matings, the spermatozoa from both *spe-8* and *snf-10* sperm had regional specific SPE-6 patterns: spermatozoa in the uterus had SPE-6 in both the pseudopod and perinuclear halo whereas those in the spermatheca had SPE-6 exclusively in the pseudopod. Therefore, neither the SPE-8 signaling pathway nor SNF-10 are absolutely required for these microenvironment specific patterns of SPE-6 localization nor is the SPE-6 pattern a reflection of activation by specific activation pathway.

We next questioned whether MO fusion during sperm activation is involved in SPE-6 localization to the pseudopod. MO fusion is a defining irreversible step in sperm activation. In this step, important sperm proteins are deposited on the cell membrane, establishing distinct polarized micro domains on the cell body and pseudopod (Figure 9D). We questioned whether polarity established by the fusion of MOs was required for SPE-6 polarization to the pseudopod. To explore this question, we analyzed SPE-6 localization in activated

*fer-1(hc1)* spermatozoa. *fer-1(hc1)* sperm are incapable of MO fusion, but respond to activation signals by polarizing and forming defective stubby pseudopods (Ward et al., 1981). This phenotype implies that some aspects of sperm activation do not require MO fusion. Examination of *fer-1* spermatozoa precociously activated within the seminal vesicle of mated males revealed that SPE-6 localized in a normal perinuclear/pseudopod pattern, similar to *swm-1*, despite the absence of MO fusion (Figure 9E). To further address a role for MO fusion in SPE-6 localization, we analyzed SPE-6 localization in *spe-46(hc197)* sperm. *spe-46* encodes a novel transmembrane protein involved in MO dynamics, and like the *spe-6* precocious activation alleles, *hc197* was identified in screens for suppressors of the *spe-8* pathway (Liau et al., 2013). *spe-46* males contain a mixture of spermatids and spermatozoa within their seminal vesicles. Importantly, about 40% of these spermatids contain precociously fused MOs, an event which is hypothesized to drive the precocious activation phenotype. Using the MSP pattern to distinguish between spermatids and spermatozoa, we found that in *spe-46(hc197)* mature spermatids that had not precociously activated, SPE-6 either localized to the perinuclear halo or was both in the perinuclear halo and cytosolic (Figure 9F, purple arrowheads), which may imply that precocious MO fusion is sufficient to induce SPE-6 to leave its perinuclear localization pattern. Unfortunately, no effective method exists to visualize MO fusion in cells that have been fixed for immunofluorescence, so we cannot be certain that it is the cells with fused MOs that have the cytosolic SPE-6. Conversely, the precociously activated *spe-46(hc197)* spermatozoa had SPE-6 in a normal

perinuclear/pseudopod (yellow arrows) or pseudopod only (pink arrowheads) localization pattern, implying that the precocious activation phenotype of *hc197* does not bypass the need for SPE-6 to relocate from an exclusively perinuclear pattern (Figure 9F). Taken together, these genetic studies suggest that MO fusion is not necessary, but may be sufficient, for SPE-6 removal from the perinuclear halo in activated spermatozoa.

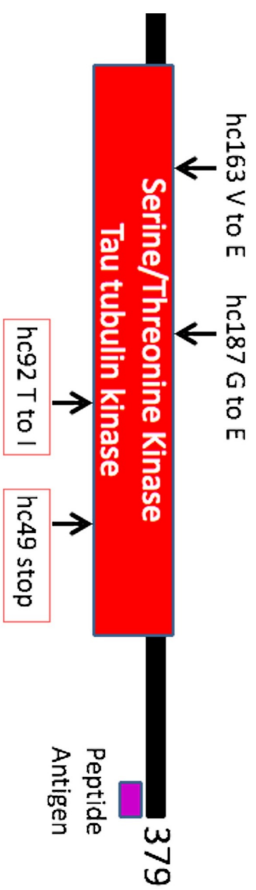
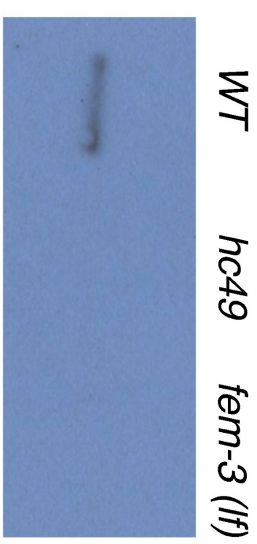
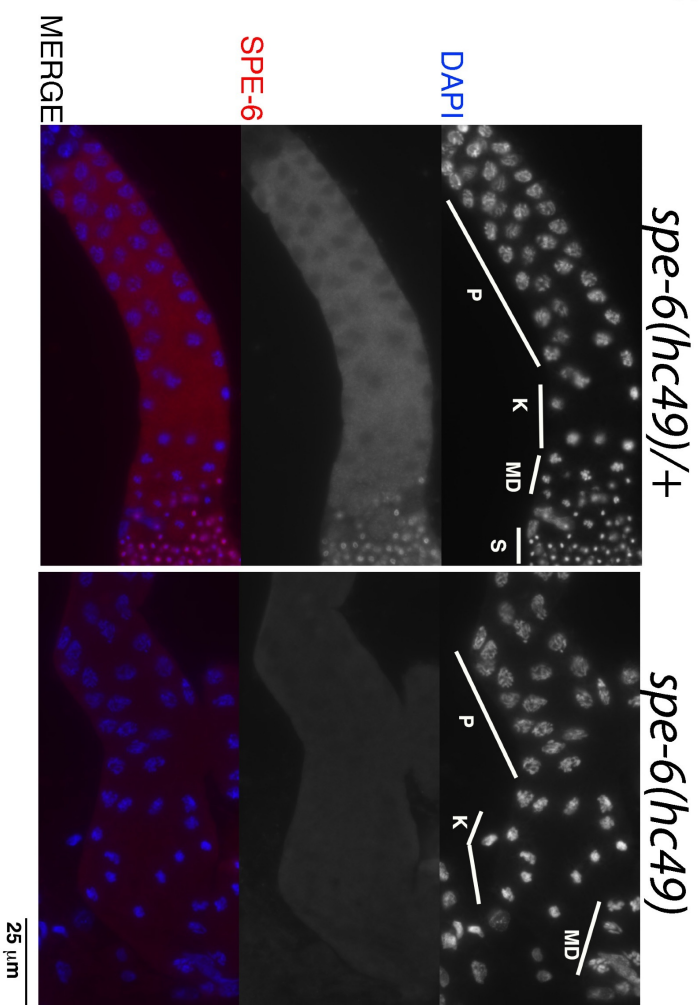
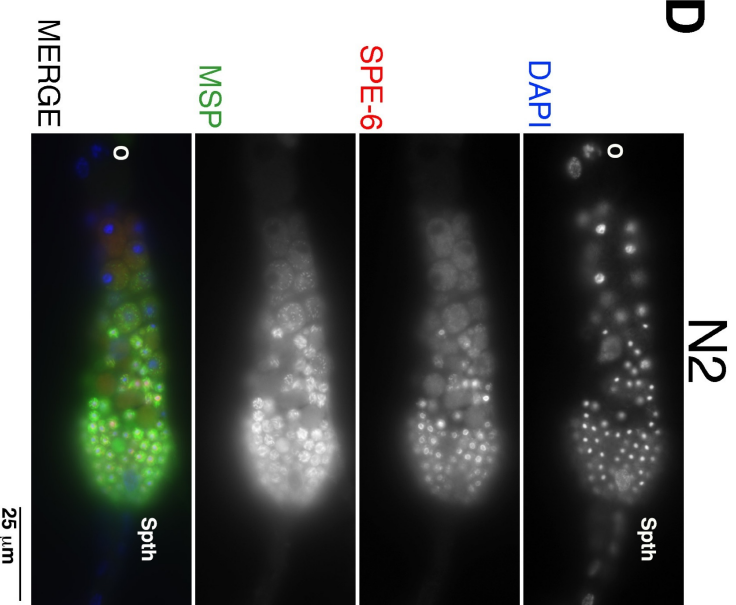
**SPE-6 is phosphorylated in spermatids and may be de-phosphorylated when localized to the pseudopod:**

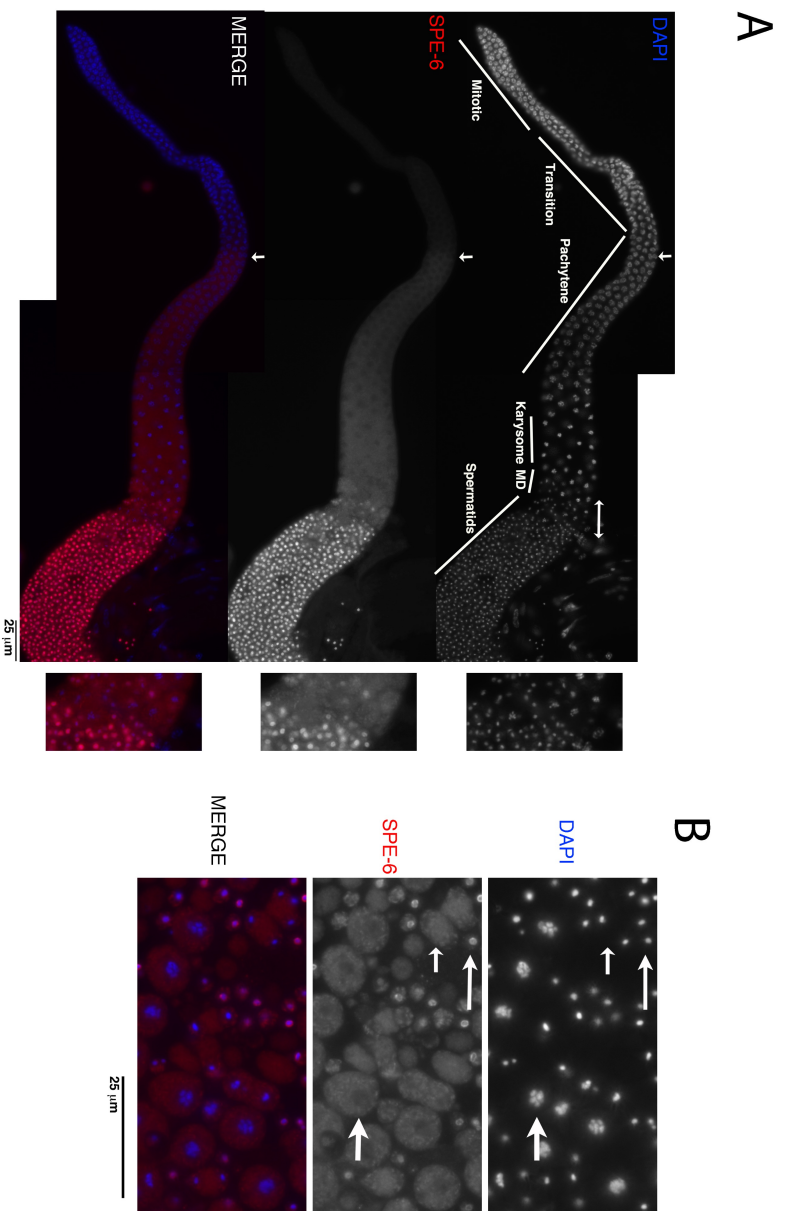
To further explore the function of SPE-6 in activated spermatozoa, we compared the post-translational modification state of SPE-6 in celibate wildtype males (unactivated spermatids) to that of *swm-1* and *spe-6(hc163)* animals (precociously activated spermatozoa). Intriguingly, we detected a second, higher mobility, band in *swm-1* and *spe-6(hc163)* lanes that was absent from an equivalently loaded wild type sample (Figure 10A, red arrow). The Casein Kinase 1 family of proteins, including the mammalian Tau Tubulin Kinase 2, are routinely regulated by protein phosphorylation, either through autophosphorylation or through the action of other protein kinases (Knippschild et al., 2014; Čajánek and Nigg, 2014). We therefore questioned whether differential SPE-6 phosphorylation might be responsible for our observed protein mobility shift. To address this question we incubated crude lysate from spermatid enriched *fem-3(q20ts)* masculinized hermaphrodites with Lambda protein phosphatase (NEB). Indeed,

treatment with Lambda phosphatase was sufficient to drive SPE-6 towards the lower molecular species in a concentration dependent manner(Figure 10B). This result is consistent with SPE-6 being phosphorylated in spermatids, and the lower molecular weight representing an un-phosphorylated protein species. These data may be consistent with a model where SPE-6 localization back and forth from the perinuclear halo to the pseudopod is regulated by the action of protein kinases or phosphatases.

**Figure 1: Construction of an affinity purified polyclonal antibody specific to SPE-6.**

(A) Cartoon schematic including the site of the peptide used for antibody generation and mutant *spe-6* alleles used here and later in this paper. (B) Western blot of SPE-6 in *him-8* and *spe-6(hc49)* males and *fem-3(e2006)* feminized hermaphrodites. (C) Epifluorescence micrographs of *spe-6(hc49)* heterozygote male and *spe-6(hc49)* homozygote male gonads. Gonads were stained with DAPI (blue) and SPE-6 (red). Animals were dissected and processed on the same slide and imaged with identical exposures. Progression of spermatogenesis labeled by zone: pachytene (P), karyosome (K), meiotically dividing (MD); haploid spermatids (S). In *spe-6(hc49)* males, meiotic dividing spermatocytes arrest in pro-metaphase and fail to make haploid spermatids. (D) Immunofluorescence micrographs of a hermaphrodite at the L4-Adult molt stained with SPE-6 (red), the Major Sperm Protein (MSP, green), and DAPI (blue). "O" labels oocyte nucleus and "Spth" marks the hermaphrodite spermatheca. In all images, distal is to the left. Scale bars denote 25µm.

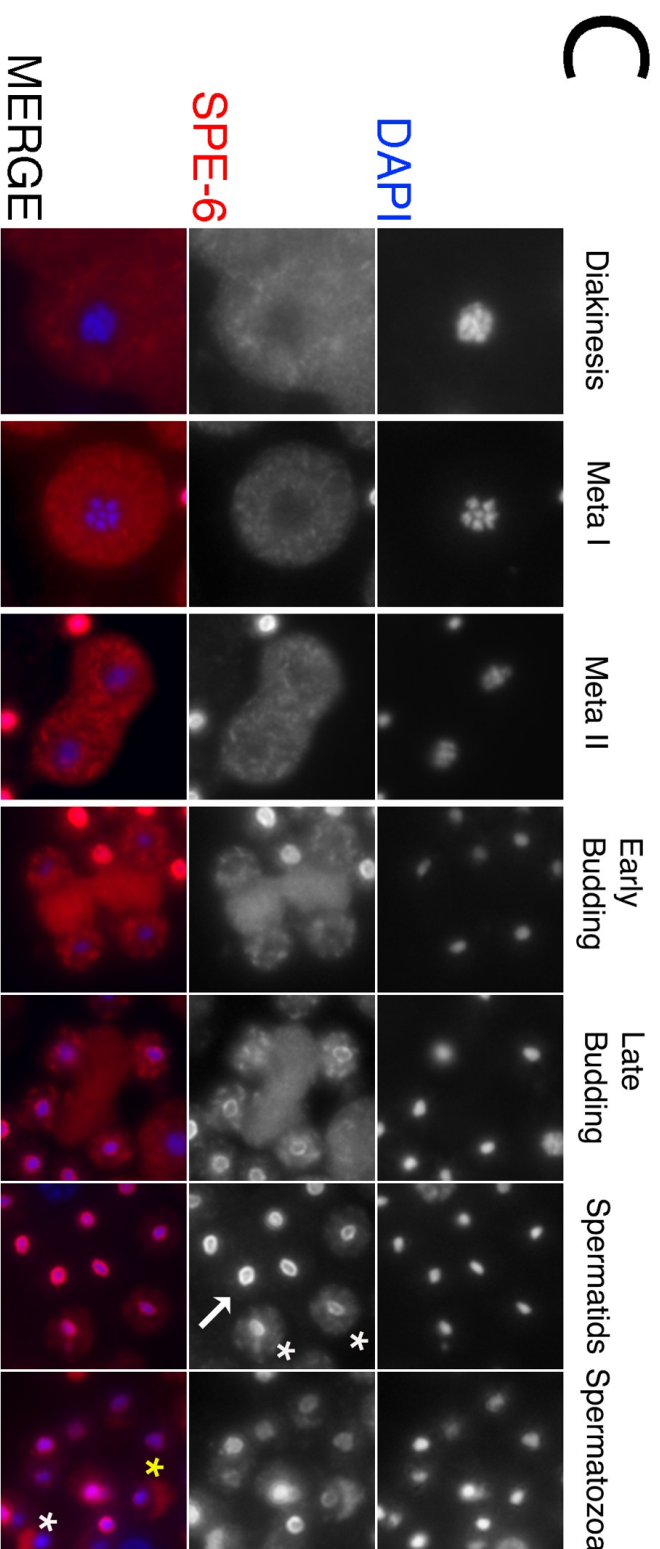
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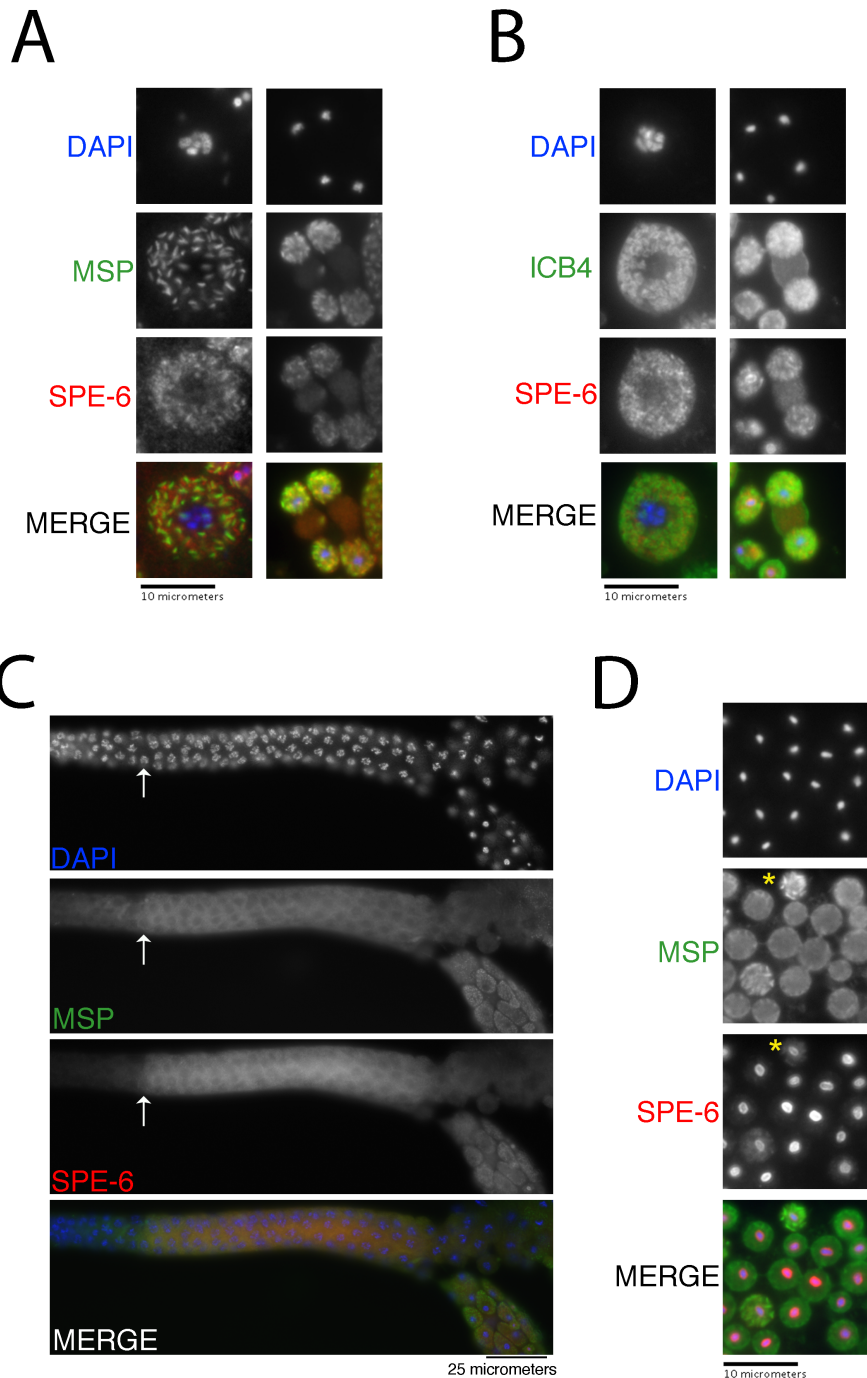
**Figure 2: SPE-6 localization changes during sperm development:**

(A) Epifluorescence micrograph of intact wild type male gonad. DAPI-stained DNA (Blue) and SPE-6 (Red). Regions of the gonads are labeled. MD (meiotic division zone). Region below double-headed arrows is enlarged 2x at the right to show granular pattern in the spermatocytes and the perinuclear pattern in the spermatids. (B) Enlarged micrograph of meiotic spermatocytes and post-meiotic spermatids. Spermatocyte (large arrow). Very early budding figure (small arrow). Spermatid (Long arrow).



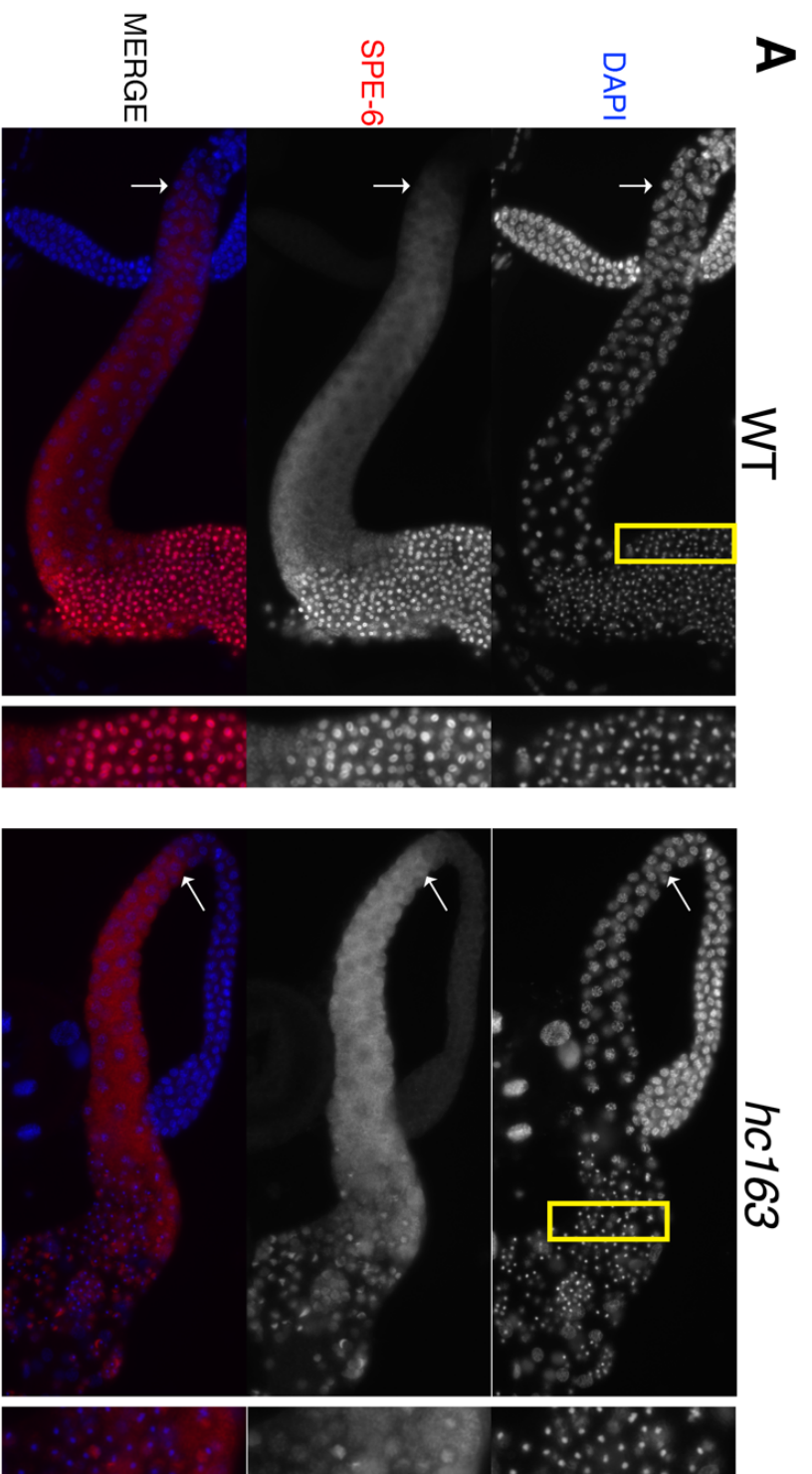


**Figure 2:** (C) Enlarged cutouts of SPE-6 localization throughout spermatogenesis. In spermatid image, asterisks show spermatids with both cytoplasmic and perinuclear SPE-6 labeling. Arrow indicates spermatid with exclusively perinuclear SPE-6 labeling. In spermatozoa image, spermatozoon to the right of the yellow asterisk has both pseudopod and perinuclear SPE-6. Spermatozoon to the right of the white asterisk has SPE-6 primarily in the pseudopod.

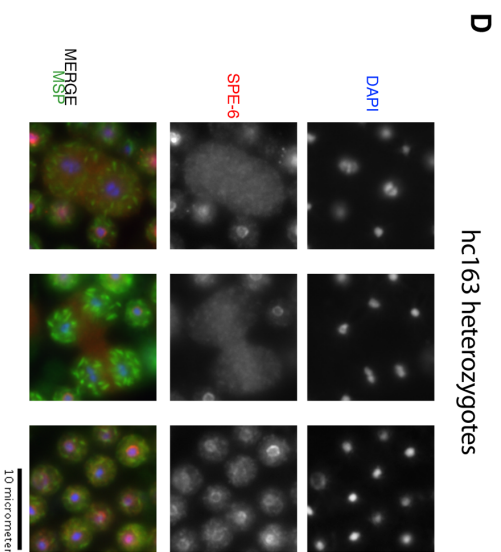
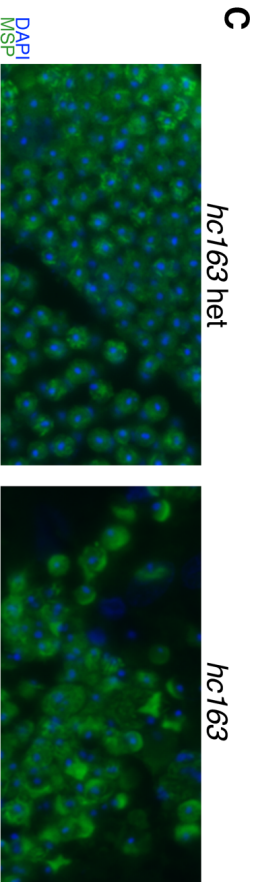
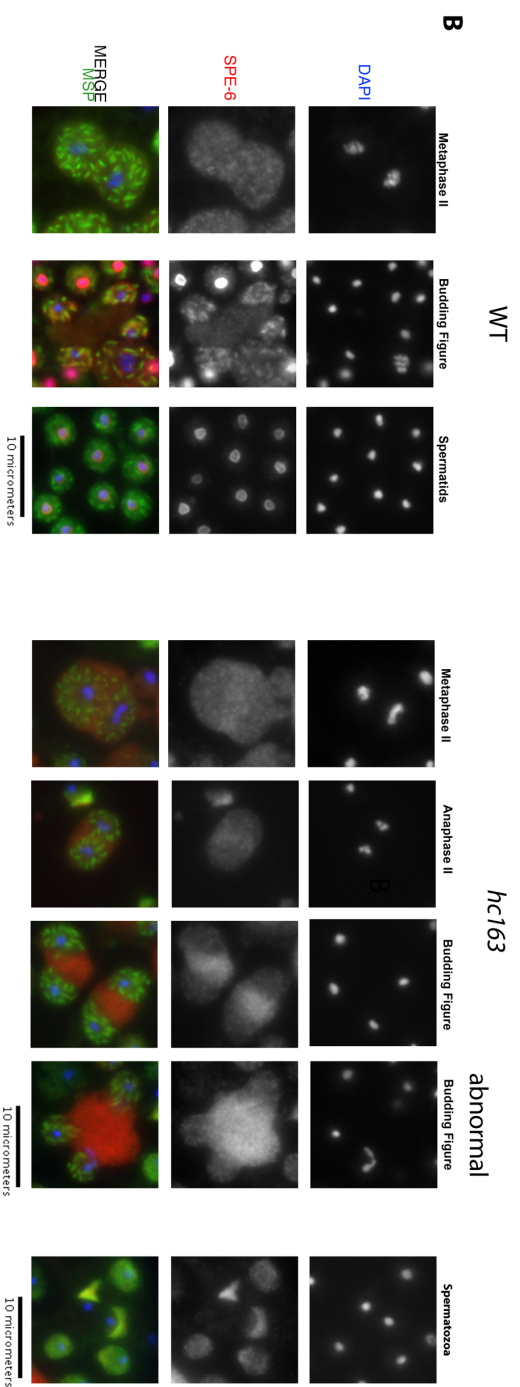


**Figure 3: SPE-6 expression and localization do not correspond to FB assembly, but do correspond to FB breakdown**

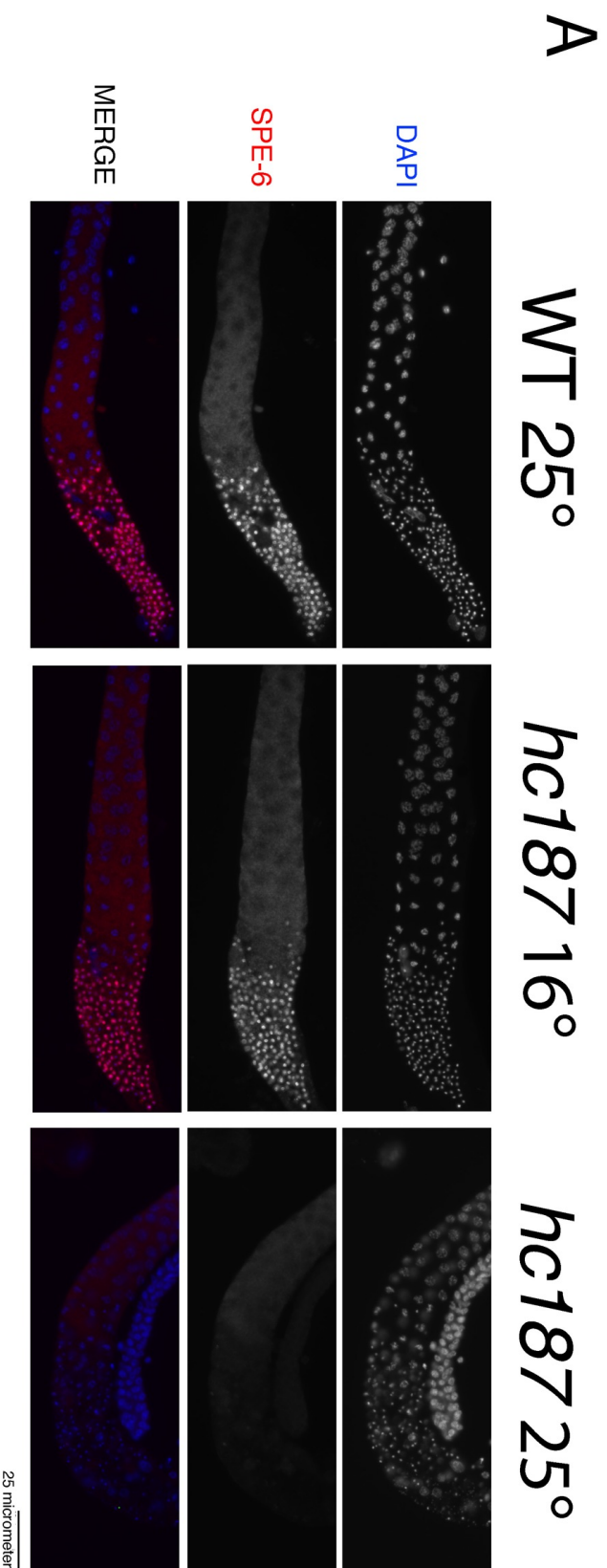
(A) Epifluorescence micrographs of wildtype male gonad stained with SPE-6, Major Sperm Protein (MSP) and DAPI. Arrows denote spermatocytes containing fibrous bodies. Distal portion of gonad is to the left. (B) Enlarged epifluorescence micrographs of primary spermatocyte and budding figures stained with SPE-6(red) and Major Sperm Protein (MSP, green). (C) Enlarged epifluorescence micrographs of primary spermatocyte and budding figures(bud) stained with SPE-6(red) 1CB4(MOs, green). (D) Enlarged epifluorescence micrographs of immature and mature spermatids with immunofluorescence against SPE-6(red) and MSP(green). All images were stained with DAPI(blue). Scale bar denotes 10 $\mu$ m.



**Figure 4: *spe-6(hc163)* produces a mislocalized protein product, but is phenotypically recessive:**  
 (A) Epifluorescent micrographs of wild type and *spe-6(hc163)* male gonads labeled with DAPI(blue) and SPE-6(red). Distal portion of gonad is to the left. Arrows show position of initial SPE-6 expression. Inset shows enlarged spermatids from boxed area of gonad.



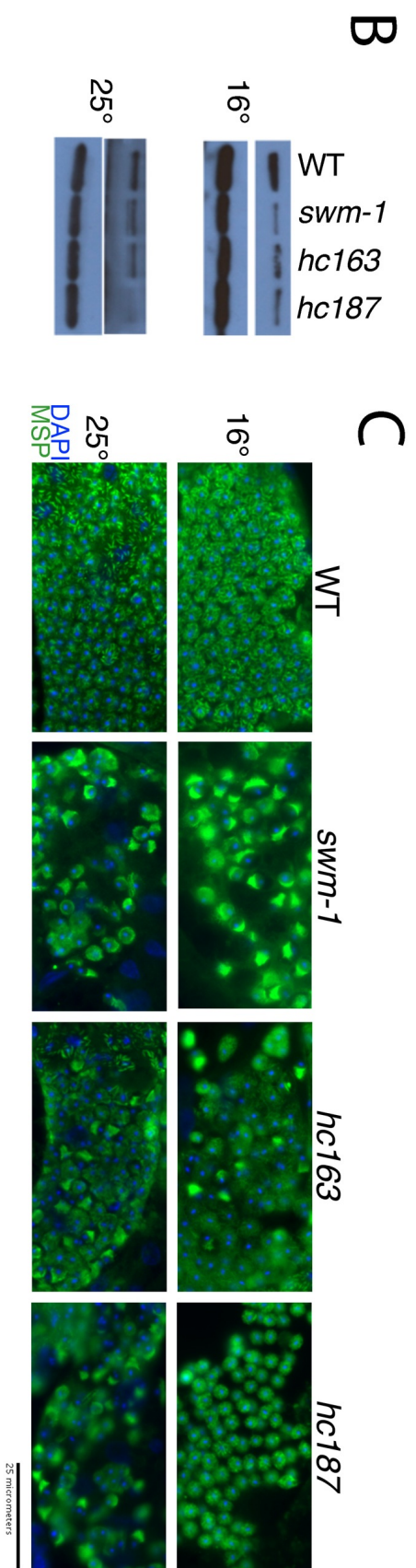
**Figure 4: 1** (B) Enlarged micrographs of wild type and *spe-6(hc163)* meiotic spermatocytes and spermatids. "Abnormal marks a budding figure with chromosome segregation defects, a common defect observed in this mutant. (C) Representative micrographs of sperm from celibate *spe-6(hc163)/spe-6(+)* heterozygote and *spe-6(hc163)* homozygote males stained with DAPI(blue) and MSP(green). (D) Enlarged micrographs of *spe-6(hc163)/spe-6(+)* heterozygote spermatocyte, budding figure, and spermatids.



**Figure 5: *spe-6(hc187)* is a temperature sensitive precocious activation mutant:**

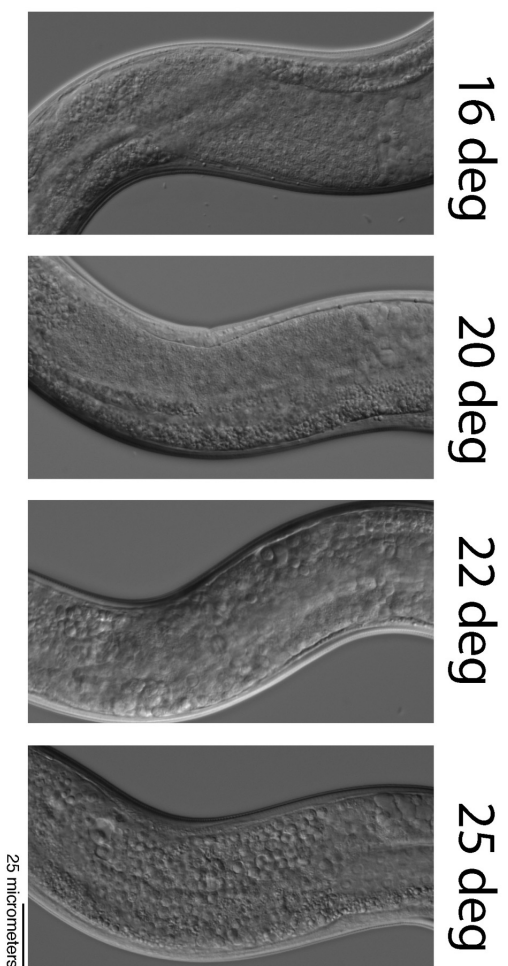
(A) Representative epifluorescence micrographs of wild type and *spe-6(hc187)* male gonads. Gonads were stained with DAPI(blue) and SPE-6(red). Males were raised from embryos or larval stage 1(L1) at the indicated temperature, picked separate from hermaphrodites as L4s to ensure celibacy, and processed for immunofluorescence at the same time. Wildtype image was taken at an exposure of 1200 ms while both *spe-6(hc187)* images were taken with an exposure time of 1500 ms.





**Figure 5:** (B, C, and D) Analysis of *him-8*(WT), *swm-1*(*me87*), *spe-6*(*hc163*), and *spe-6*(*hc187*) males. Animals were raised at the indicated temperature from embryos or L1 stage. (B) Western blots of wild type and mutant whole worm lysates from 80 similarly aged worms. Top insets are films from membrane probed with anti-SPE-6 at an approximate MW of 42 KDa. Bottom film insets are membrane fragments from the same gel probed with anti-MSP, bands are at approximate MW of 15 KDa. MW determined with kaleidoscope protein standards. (C) Epifluorescence micrographs of representative sperm spreads from celibate males stained with DAPI(blue) and MSP(green) (D) Gonads of celibate males were scored for the presence or absence of activated spermatozoa. Each gonad was categorized as containing less than 1% (blue), between 1 and 5% (green), and greater than 5% (yellow) activated spermatozoa. N values are displayed in parentheses within each bar.

A



**Figure 6: *spe-6(hc187)***

**phenotype at**

**intermediate**

**temperatures:**

(A and C) celibate

*spe-6(hc187)* males were

raised at the indicated

temperature. (A) DIC

micrographs of the seminal

vesicle of whole mounted

males raised at the

indicated temperature. (C)

Epifluorescent micrographs

of sperm spreads from

sibling worms from (A).

Gonads were stained for

SPE-6(red), Major Sperm

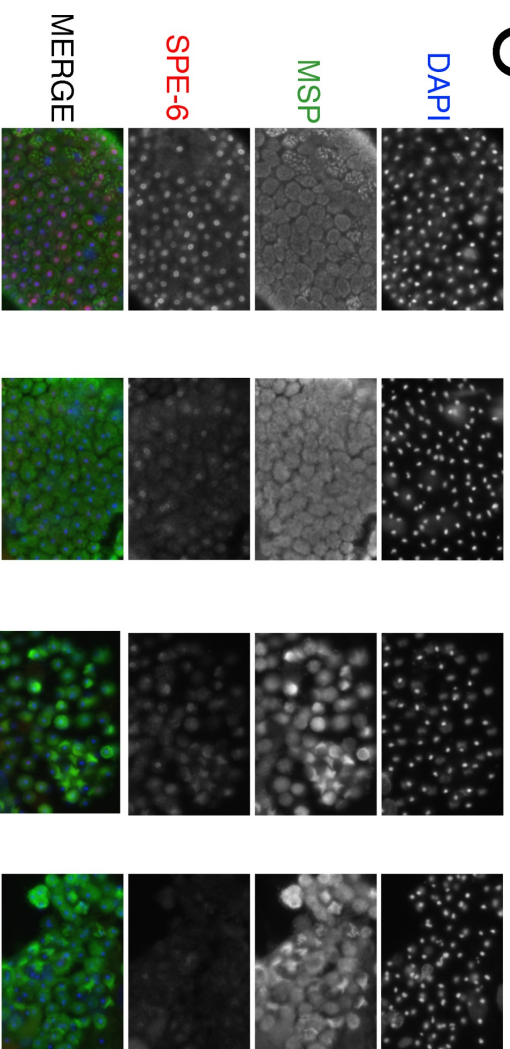
Protein(MSP, green), and

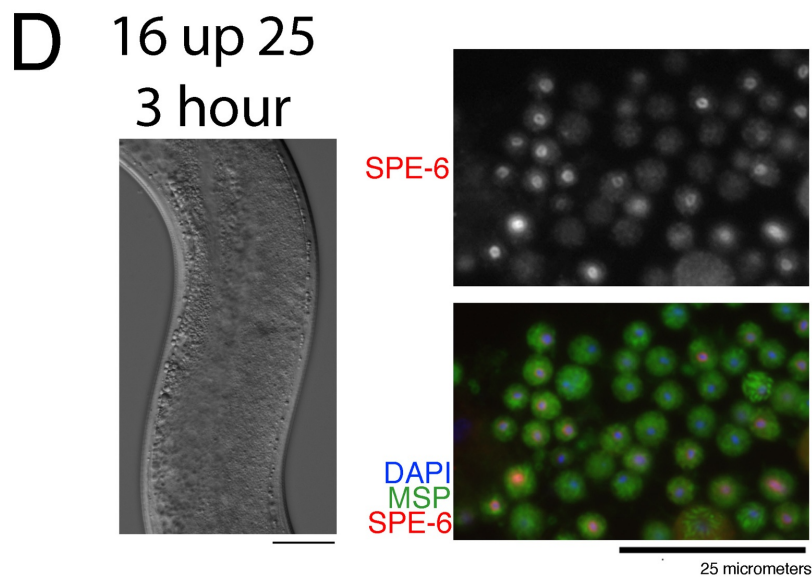
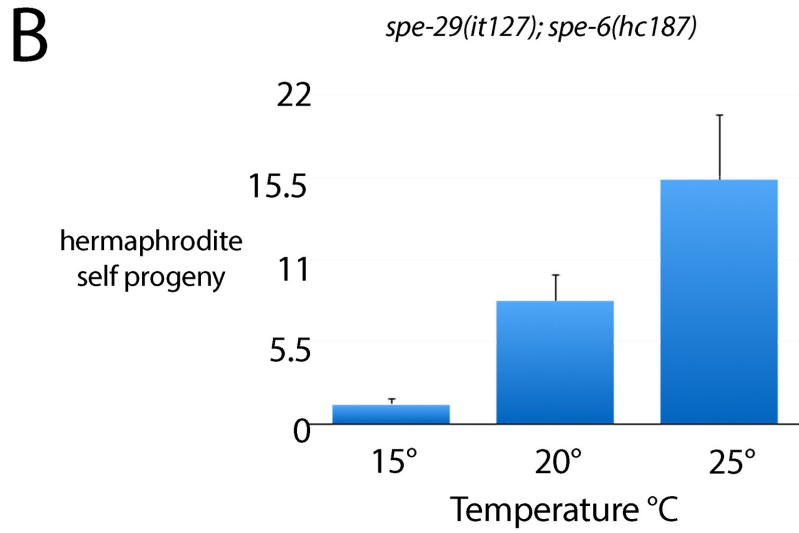
DAPI(blue). SPE-6

exposures were not

standardized.

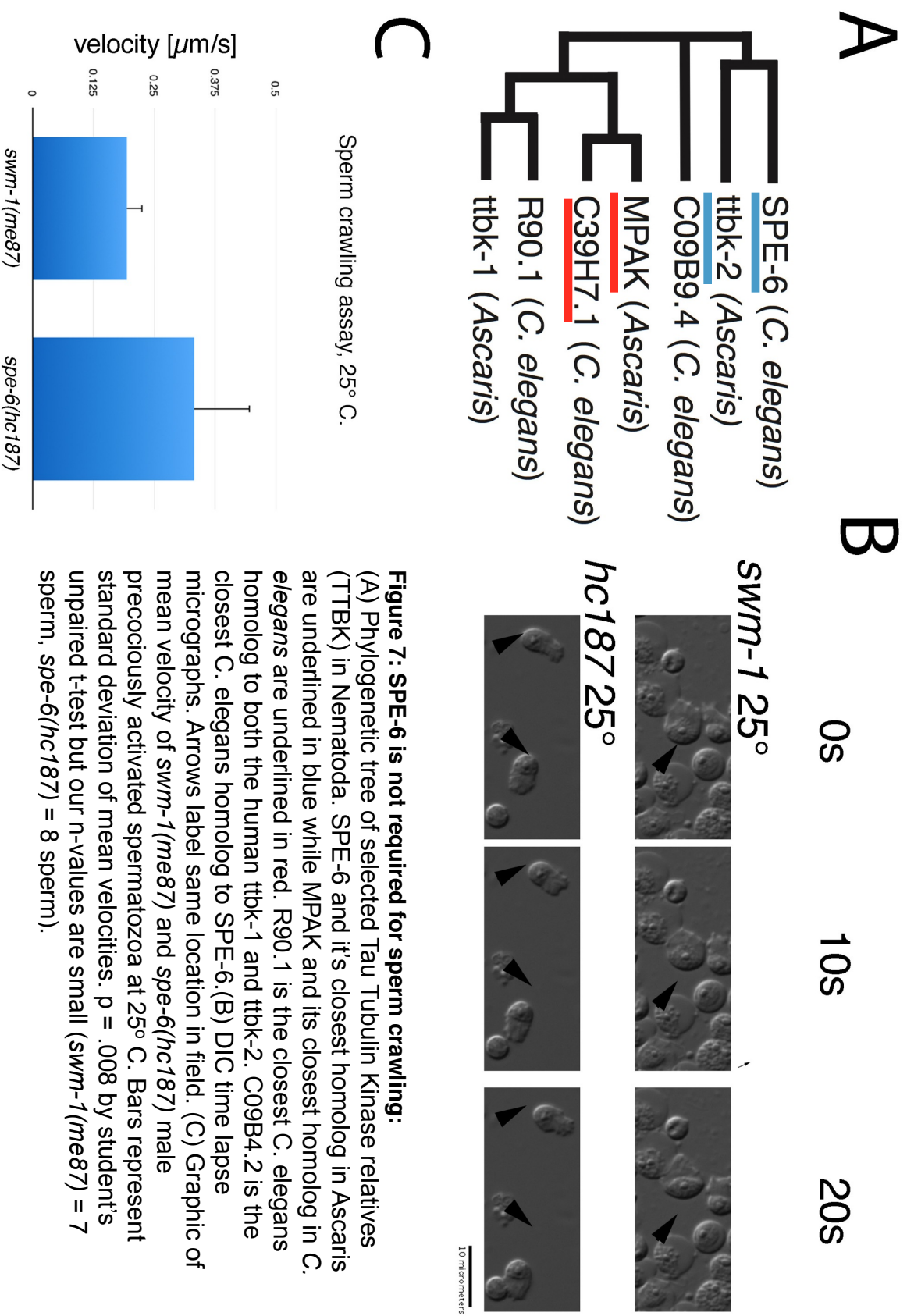
C





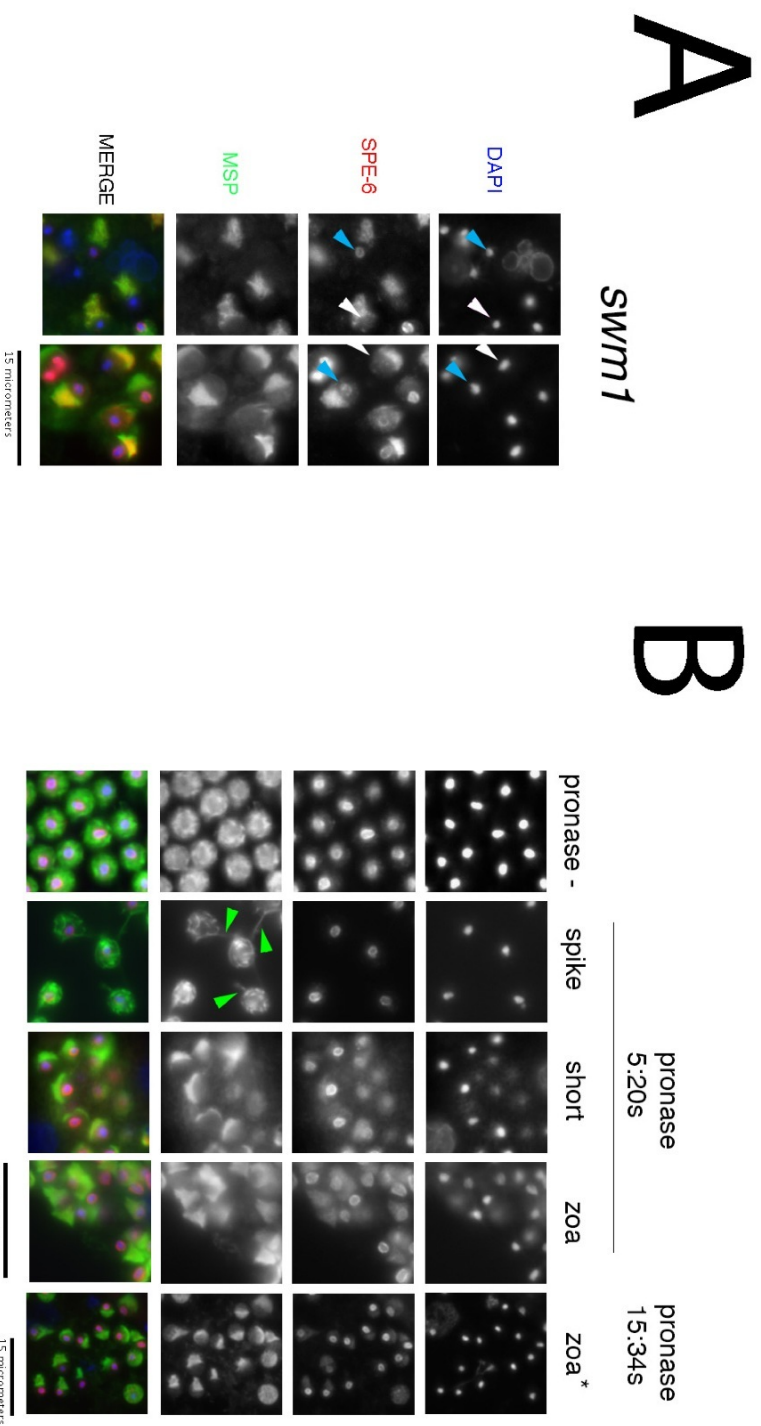
**Figure 6:** (B) Fertility measurements of *spe-29(it127);spe-6(hc187)* hermaphrodites raised at the indicated temperatures. For context, normal hermaphrodite fertility self fertility counts are near 300. (D) DIC and epifluorescence micrographs of sibling celibate *spe-6(hc187)* males raised at 16 and upshifted to 25degrees for three hours. Sperm were stained with SPE-6(red), MSP(green), and DAPI(blue).





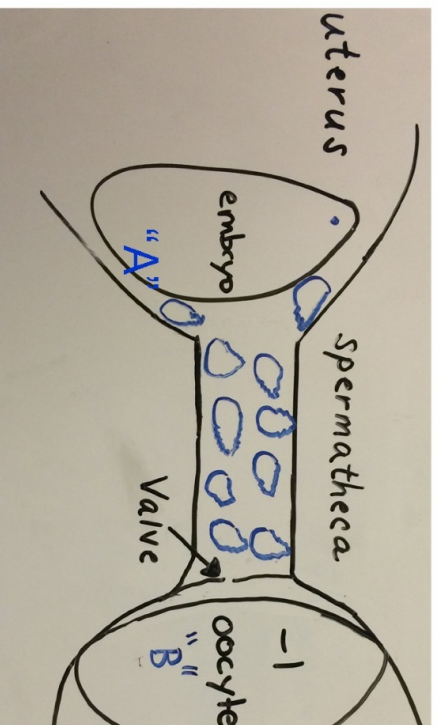
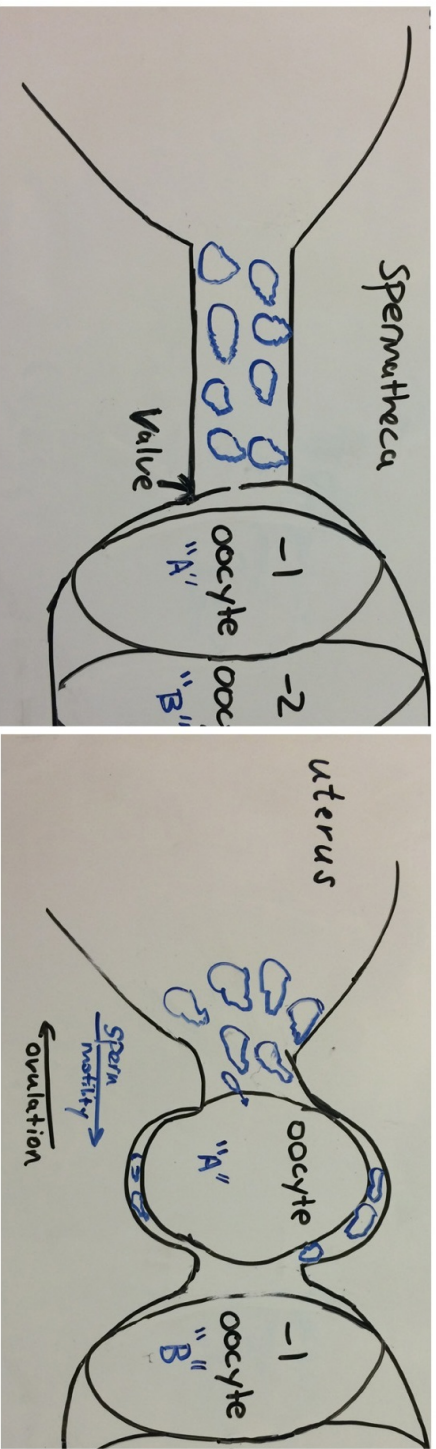
**Figure 7: SPE-6 is not required for sperm crawling:**

(A) Phylogenetic tree of selected Tau Tubulin Kinase relatives (TTBK) in Nematoda. SPE-6 and its closest homolog in *Ascaris* are underlined in blue while MPAK and its closest homolog in *C. elegans* are underlined in red. R90.1 is the closest *C. elegans* homolog to both the human ttbk-1 and ttbk-2. C09B4.2 is the closest *C. elegans* homolog to SPE-6. (B) DIC time lapse micrographs. Arrows label same location in field. (C) Graphic of mean velocity of *swm-1(me87)* and *spe-6(hc187)* male precociously activated spermatozoa at 25° C. Bars represent standard deviation of mean velocities.  $p = .008$  by student's unpaired t-test but our n-values are small (*swm-1(me87)* = 7 sperm, *spe-6(hc187)* = 8 sperm).

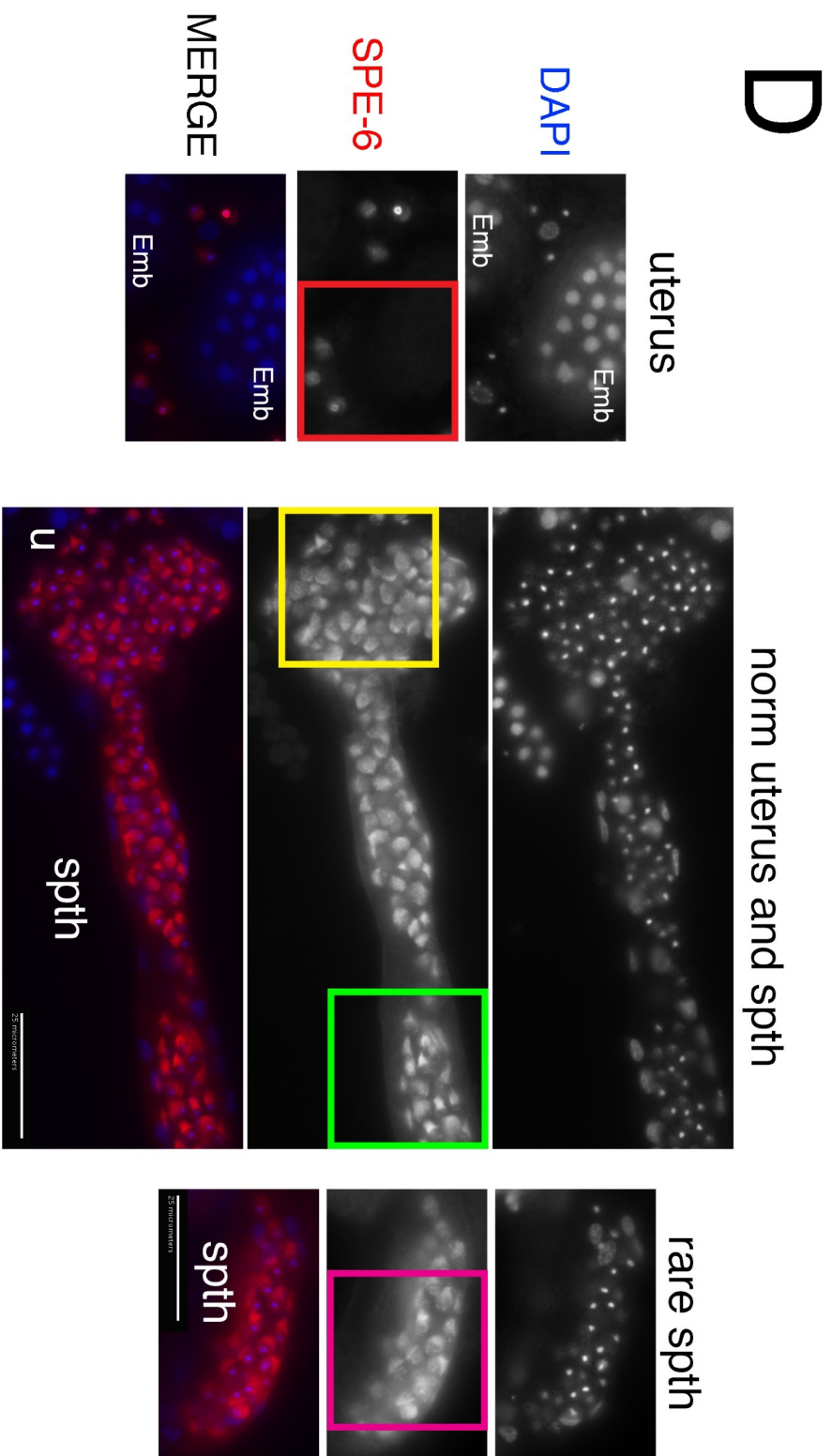


**Figure 8: Context dependent SPE-6 localization in activated sperm:**(A-D) Epifluorescent micrographs of *C. elegans* sperm. In merge images, DAPI (blue) SPE-6(red) and MSP (green) (A) Representative micrographs of precociously activated spermatozoa from *swm-1(me87)*. Blue arrowheads point to perinuclear SPE-6 in spermatozoa with extended MSP and SPE-6 labeled pseudopods. Pink arrowheads point to in-focus chromatin masses in spermatozoa that have SPE-6 labeled pseudopods but lack perinuclear SPE-6. (B) Micrographs of wild type male sperm dissected and frozen in the absence (pronase -) or presence of the *in vitro* sperm activator pronase. Images labeled “spike,” “short,” and “zoa” indicate sperm at different stages in activation sequence include the spike stage, the initial emergence of short under-developed pseudopods, and mature spermatozoa with full pseudopods. Green arrowheads label MSP-containing spikes. \* Denotes an image taken at a lower magnification than the others.

C



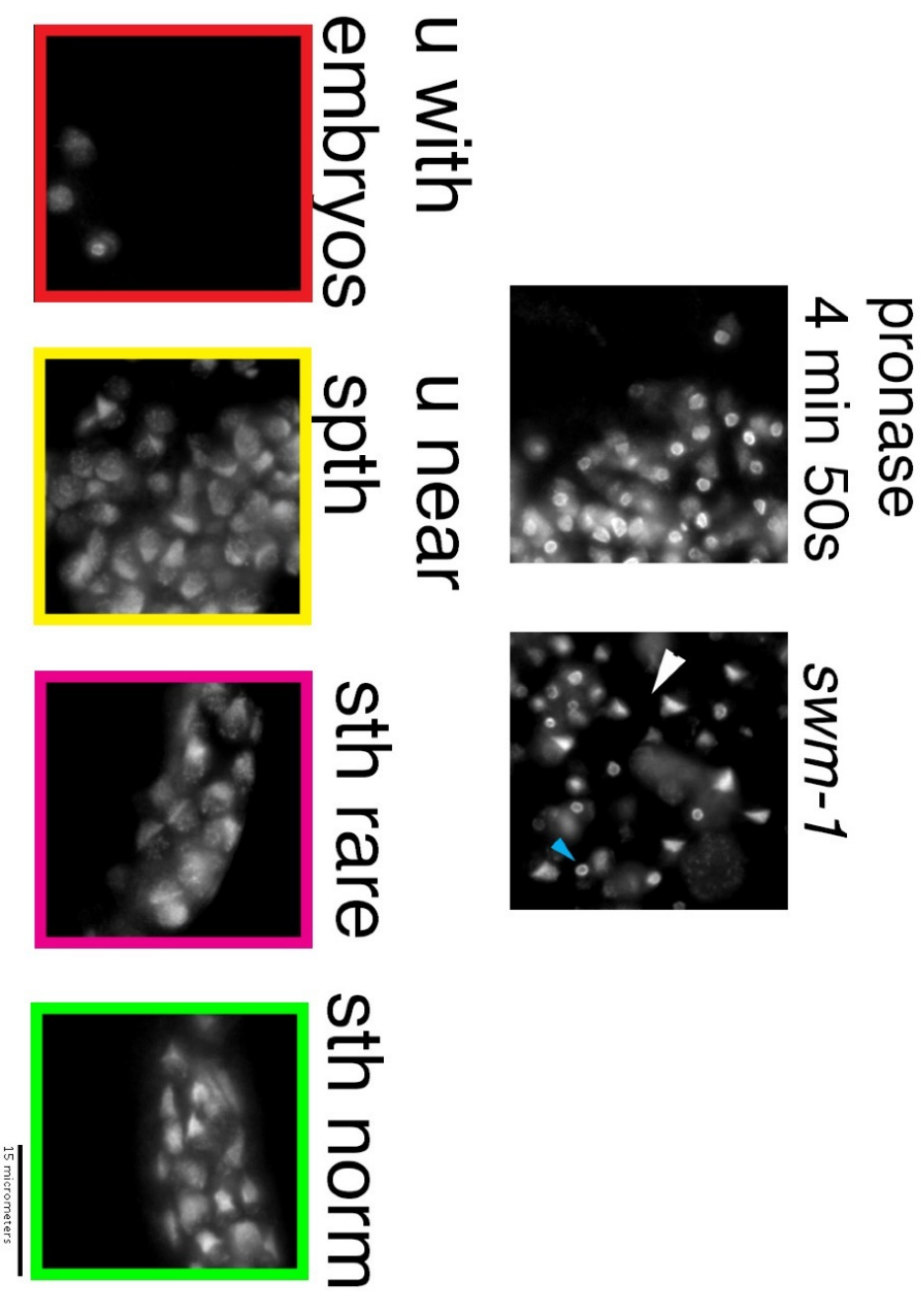
**Figure 8:** (C) Line drawing of sperm motility and ovulation at the hermaphrodite spermatheca. Activated spermatozoa (blue) wait in the spermatheca for the passage of an oocyte. During ovulation, oocytes pass through the spermatheca and are fertilized; simultaneously, spermatozoa are displaced from the spermatheca. Sperm must re-enter the spermatheca to be competent for fertilization of the next oocyte.



**Figure 8:** (D) Epifluorescent micrographs of male sperm within the reproductive tract of *fog-2(q71)* “females”. Position of sperm among later stage embryos implies more recent sperm entry (“uterus”, left). “u” and “sth” denote the uterus and spermatheca respectively. Representative (“norm”) and non-representative (“rare”) spermathecae are shown.

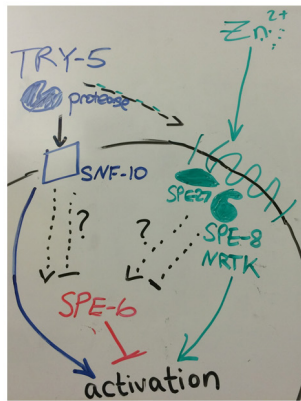


# E

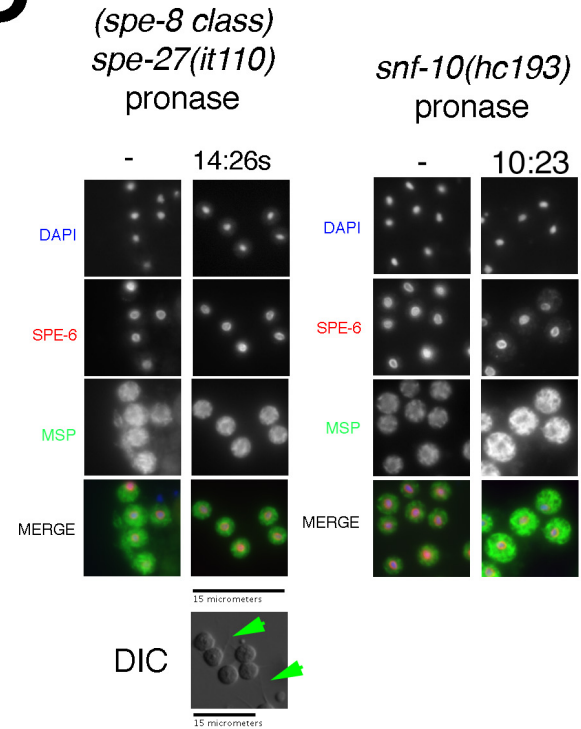


**Figure 8:** (E) SPE-6 patterns from images not shown and boxed regions from (D) were enhanced by using Adobe Photoshop to purposely discard low intensity information and accentuate differences in SPE-6 localization patterns.

# A



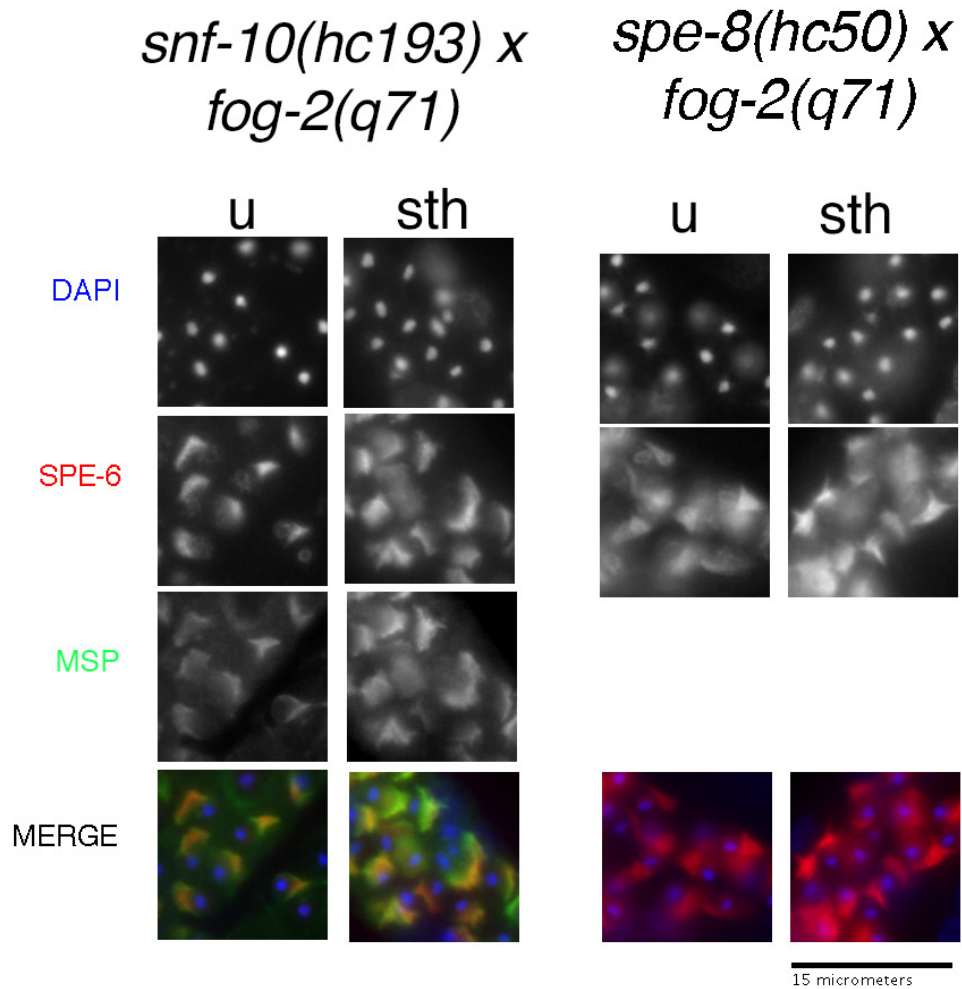
# B



## Figure 9: SPE-6 localization in sperm activation mutants:

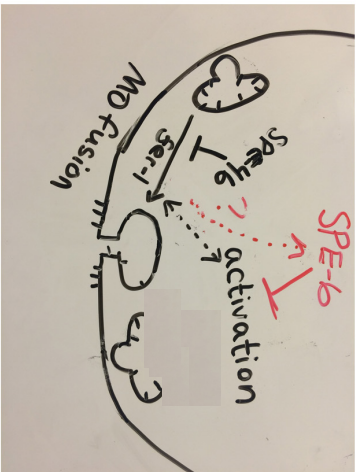
(A) Cartoon of extracellular and membrane bound sperm activation pathway components. Arrows indicate genetic rather than physical interaction. “NRTK” stands for Non-Receptor Tyrosine Kinase, the identity of the *spe-8* protein product. (B, C, E, and F) Epifluorescent micrographs of sperm under different genetic, pharmacological, and physiological conditions. Sperm were stained with SPE-6(red), Major Sperm Protein (green), and DAPI(blue). (B) Micrographs of *spe-27(it110)* (a member of the *spe-8* class of sperm activation genes) and *snf-10(hc197)* sperm dissected in the presence of the pronase *in vitro* activator. “DIC” labels a DIC micrograph of the same cells as above, acquired prior to freezing and fixation. Green arrow in DIC image labels arrested spike stage of *spe-8* class sperm. Numbers indicate the amount of time sperm were left in pronase before freezing for immunofluorescence, “-” denotes pronase free control.

C

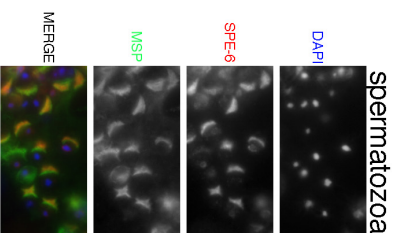


**Figure 9:** (C) Micrograph of spermatozoa activated *in vivo* acquired by analyzing male sperm in mated *fog-2(q71)* feminized hermaphrodites. “u” and “sth” denote sperm found within the uterus and spermatheca respectively.

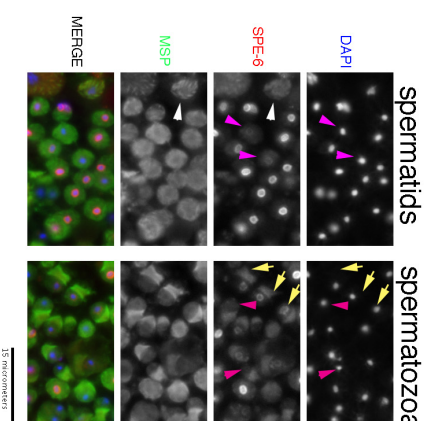
D



E

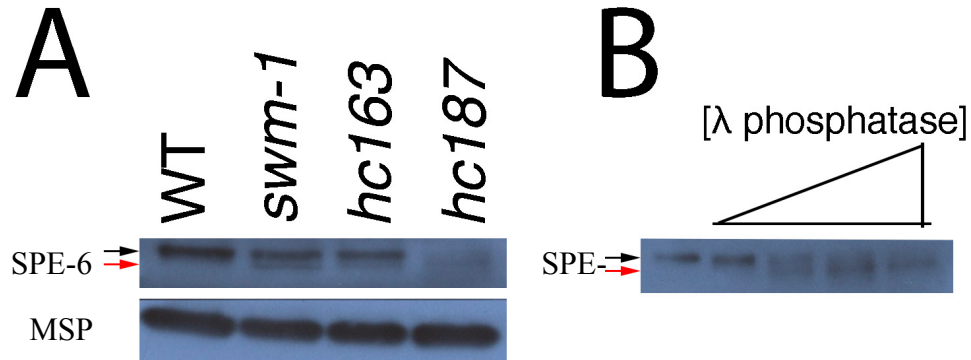
*fer-1(hc1)* SV

F

*spe-46(hc197)* SV

**Figure 9:** (D) Cartoon of FER-1 and SPE-46 involvement in MO fusion and a possible interaction with SPE-6. FER-1 is required for MO fusion and SPE-46 is required to prevent precocious MO fusion. MO fusion is thought to release important membrane bound proteins to the cell surface essential to proper motility and the establishment of polarized domains in the spermatozoon. (D and E) Micrographs of *fer-1* and *spe-46(hc197)* spermatozoa. (E) *fer-1* males were raised at a restrictive 25° C and allowed to mate for 3 days in order to produce ample numbers of spermatozoa. Immature spermatids (white arrowhead), mature spermatid(White) precociously activated spermatozoa. Epifluorescent micrographs of *spe-46(hc197)* spermatids and cytosolic SPE-6 in mature spermatid (purple), spermatozoa with SPE-6 exclusively in pseudopod (magenta), and spermatozoa with SPE-6 in both the pseudopod and the perinuclear halo (yellow) are all labeled with arrows.





**Figure 10: Evidence for phosphorylation of SPE-6**

(A) SPE-6 (top, MW:43 kDA) and Major Sperm Protein (MSP, bottom, MW: 15kDA) western blots of wild type, *swm-1(me87)*, *spe-6(hc163)*, and *spe-6(hc187)* males raised at 25° C (Same blot as in figure 5, but replicates have been completed and are in progress). Each lane contains equivalent of lysate from 40 adult males. MSP functions as sperm specific loading control. Red arrow points to higher mobility band. (B) Western blot of *fem-3gf(q20ts)* masculinized hermaphrodite lysate incubated with increasing concentrations of Lambda ( $\gamma$ ) phosphatase (NEB) in parallel. Numbers denote numbers of units of phosphatase in each sample. Red arrow points to the band driven by increasing concentration of Lambda phosphatase. Representative blot from one of two experiments.

## **Discussion:**

Nematode sperm are tiny streamlined cells charged with the essential role of passing on genetic information. Without actin, tubulin, or new protein synthesis, nematode sperm undergo dramatic morphological changes, respond to complex spatial environments, and fertilize oocytes with remarkable efficiency. This process begins in meiotic spermatocytes with the formation of FB-MOs which ensure polarized segregation of the motility protein MSP into haploid spermatids. A second key step is sperm activation, where sperm undergo irreversible changes to acquire new cellular properties including the acquisition of motility and the capability to fertilize oocytes. Mutant sperm incapable of activation cannot fertilize oocytes while mutants with precociously activated sperm retain hermaphrodite but lose male fertility. In nematodes, sperm must also be prepared for the long haul; after insemination, they do not just have one opportunity for fertilization but the “assembly line of oocytes” gives them multiple opportunities to participate in a fertilization event. Here we have described the cytological details of the SPE-6 protein kinase previously identified as a key regulator of both of these processes. We found that SPE-6 has a complex and dynamic localization pattern that shifts localization at key steps following sperm individualization. In addition, we have strengthened the argument that SPE-6 inhibits sperm activation and implicated the sperm perinuclear halo as an unexpected compartment for regulation of sperm activation. However, our results point to a function for SPE-6 beyond regulation of the irreversible process of

sperm activation and implicate SPE-6 as a dynamic regulator of spermatozoa interaction with the extracellular environment.

Within developing spermatocytes, the wild type localization of SPE-6 protein revealed surprisingly few clues to its function in the formation of fibrous bodies. While cytosolic SPE-6 would still be capable of interacting directly with the MO machinery to promote or maintain fibrous body stability, the observation that SPE-6 does not localize directly to the FB-MOs raises the possibilities that SPE-6 may function indirectly to promote FB-MO formation or that SPE-6 dynamically localizes to the FB-MO complex without actually accumulating there. In either case, superficially normal FB formation occurs in *spe-6(hc187)* developing spermatocytes (data not shown) implying that SPE-6 is required only in catalytic amounts for fibrous body formation. Furthermore, analysis of null alleles of both *spe-6* and another gene required for FB-MO formation suggest that the *spe-6* null mutation may have a pleiotropic effect on other aspects of early spermatogenesis outside of FB-MO formation (Kari Price and Diane Shakes, unpublished).

At stages following the meiotic divisions, the localization pattern of SPE-6 is more informative. At this point, SPE-6 experiences distinct cytoplasmic relocation during three important transitions: cellularization of spermatids during the budding division, fibrous body breakdown in spermatids, and sperm activation. Many proteins involved specifically in early processes of *C. elegans* spermatogenesis are degraded or discarded at this point, therefore the persistence and dynamic localization of SPE-6 points to a molecular function

specifically in post-meiotic sperm. The function of SPE-6 in spermatids is complicated by the localization of SPE-6 to a perinuclear compartment, rather than the cell membrane or membrane docked MOs which are the location of all other identified regulators of sperm activation.

Evidence that SPE-6 is genetically recessive led to a model where SPE-6 functions in the spermatid to inhibit sperm activation, with genetic loss of SPE-6 causing precocious sperm activation (Muhlrad and Ward, 2002) (Liau et al., 2013). The function of SPE-6 as a negative regulator of sperm activation is confirmed by protein level analysis of *spe-6(hc187)* determining that temperature sensitive loss of SPE-6 protein can drive a precocious activation phenotype. Our analysis of the localization defects of *spe-6(hc163)* also help explain why *hc163* is the most robust suppressor allele of *spe-27(it132)*. By only discarding SPE-6 protein after the meiotic divisions, *spe-6(hc163)* sperm have sufficient SPE-6 for the protein's early functions within spermatocytes, yet are poised to precociously activate in spermatids which are both depleted of SPE-6 and have mislocalized the small amounts of protein that do make it into the spermatids. Our studies of these two alleles suggest that the complicated selective pressure which generated *spe-6(hc163)* may have generated other *spe-6* alleles with interesting properties.

Combined with our new localization data, a simple model of sperm activation has SPE-6 active within the spermatid perinuclear halo waiting for an inhibitory signal transduced from the cell membrane. Thus far, no candidate gene products involved in sperm activation have been identified which co-localize

perinuclearly with SPE-6. A model for how a signal could be transduced from the membrane to the cytoplasm or perinuclear halo stems from the recent report that SPE-8 (a tyrosine kinase) disassociates from the plasma membrane during sperm activation. However, SPE-8 is not absolutely required for SPE-6 localization as SPE-6 localizes normally in *in vivo* activated male spermatozoa. SPE-6 is regulated by localization changes in activated spermatozoa, implying that SPE-6 is involved in more than just a one-time momentary “off switch” during sperm activation. Instead our study points to a functional role of SPE-6 beyond the genetically defined function in the initial irreversible process of sperm activation.

The identity of SPE-6 as a Casein Kinase 1 (CK1) leaves a dizzying array of possible substrates, with a recent review listing 140 CK1 targets validated *in vitro* or *in vivo* (Knippschild et al., 2014). Stepping back from the specific proteins and processes that CK1s regulate, a more generalizable statement about CK1 function is that they tend to specifically be involved in the regulation of highly dynamic processes. Members of the CK1 superfamily themselves are often subjected to autoinhibitory phosphorylation of their C-terminal tail, and this phenomenon was also recently reported for the human version of Tau Tubulin Kinase 2. These modifications inhibit enzymatic function, but are reversed by either protein phosphatases or truncation of the C-terminal tail. Thus autoinhibition proves a tightly controlled biochemical handle for enzyme regulation. Because of this feature, CK1s are ideal for transient regulatory processes including the DNA damage response, endocytic trafficking, the

circadian clock, and as described here the regulation of sperm motility. Unlike other CK1s, human Tau Tubulin Kinase 2 preferentially phosphorylates substrates with a phosphotyrosine at the +2 position from the phosphorylation site. Since phosphotyrosine modifications are known to regulate other proteins that modulate nematode sperm motility, the coordinated modification of phosphotyrosine containing proteins may be a key aspect of SPE-6 function and regulation (Bouskila et al., 2011; LeClaire et al., 2003; Muhlrads et al., 2014).

Our preliminary finding that SPE-6 primarily exists as a phosphorylated species in *C. elegans* spermatids with increasing amounts of un-phosphorylated SPE-6 in activated spermatozoa, is consistent with SPE-6 following a similar regulatory scheme as its more thoroughly characterized TTBK and CK1 relatives. However, the idea that the phosphorylated SPE-6 present within spermatids represents the inhibited state is inconsistent with our genetic evidence that loss of SPE-6 causes precocious sperm activation. Possible interpretations of this paradox might be that only very low levels of unphosphorylated SPE-6 is required to inhibit sperm activation, or that the putative phosphorylated species of SPE-6 within spermatids is not autoinhibitory in nature. Modulation of SPE-6's phosphorylation state and sub-cellular localization might enable SPE-6 to regulate not only the initial irreversible step of sperm activation but also microenvironment responsive changes in the behavior and motility of already activated spermatozoa. One candidate for modifying the phosphorylation state of SPE-6 are the abundantly expressed sperm-specific phosphatases within nematode sperm.

What is the function of SPE-6 in sperm following the initial steps of sperm activation? SPE-6 is found in distinct context-specific localization patterns suggesting dynamic regulation of SPE-6 localization and function. The low levels of SPE-6 found in *spe-6(hc187)* sperm do not compromise motility, chemotaxis, or fertilization, implying that SPE-6 does not have a stoichiometric contribution to any of these processes. In contrast, our preliminary evidence finds that lowered SPE-6 protein levels cause an increase in sperm motility, providing tantalizing evidence that SPE-6 may function to negatively regulate not only the initial step of sperm activation but also later dynamics of sperm motility. The sperm microenvironment dependent SPE-6 localization dynamics are consistent with such a model, since sperm motility and SPE-6 both show response to unidentified stimuli in the “female” reproductive tract. If the observation that TTBK2 phosphorylates residues proximal to phosphotyrosine residues is extendable to related proteins, we postulate that SPE-6 might modulate the spermatozoon’s response to extracellular environment by direct modification of tyrosine phosphorylated MSP-associating proteins involved in motility.

A recent report described the consequences of cross-species matings in the *Caenorhabditis* clade with “heterospecific” sperm moving past the spermatheca into the feminine germ line and soma, causing tissue damage and sterilization (Ting et al., 2014). What sort of extracellular signals might be present in the spermatheca microenvironment to normally halt sperm motility and to cause the observed changes we observe in SPE-6 localization? Soluble prostaglandin signals have been implicated in positively regulating sperm

chemotaxis, and it is also known that molecular cross talk goes in the other direction, with soluble signals from the sperm influencing oocyte meiosis (McKnight et al., 2014; Miller et al., 2001). It is conceivable that diffusable signaling molecules might be concentrated in the spermatheca to halt spermatozoa movement and prevent sperm from moving past the spermatheca. However, complete restriction of a soluble signal to a domain as small as the spermatheca is likely impossible, and a signal that inhibits motility would have disastrous effects on fertility if present in the wrong spatial or temporal context.

The *C. elegans* spermatozoon contains a polarized membrane with distinct isolated membrane domains; proteins involved in processes like fertilization are specifically restricted to the pseudopod domain of the membrane (Xu and Sternberg, 2003). Perhaps the pseudopod contains other proteins involved in juxtacrine signals between the sperm and spermatheca that regulate sperm motility. In an analogous manner, divergent protein-protein interactions between cell surface receptors of sperm and oocytes prevent cross-species fertilization; a similar mechanism of molecular mismatching may prevent heterospecific (different species) sperm attachment to the spermatheca in cases of “killer sperm” (Ting et al., 2014). Unpublished transmission electron micrographs from nearly 30 years ago show complex and intimate physical interactions between *C. elegans* spermatozoa and the spermatheca (Diane Shakes and Sam Ward, unpublished data). Physical interactions between sperm and the spermathecae might elicit cell signaling within the sperm themselves leading to the intracellular changes eliciting SPE-6 localization changes. It would



be interesting to study both the effect of depletion of SPE-6 on the trafficking of spermatozoa in the feminine reproductive tract, as well as the localization of SPE-6 in other *Caenorhabditis* species during heterospecific mating experiments.

Here we have described the first report of a sub-cellular regulatory change, SPE-6 trafficking between the cell body and pseudopod, occurring after sperm have already activated. A recent report characterized a pseudokinase, COMP-1, that regulates the efficiency of *C. elegans* sperm migration, yet is localized entirely to the cell body (Hanson et al., 2015). This report, along with our data on dynamic SPE-6 localization changes in activated spermatozoa, implicates the cell body as a surprising location for control of sperm motility. Such control might be involved in both preventing precocious sperm activation and secondly regulating sperm function in the right spatial temporal context as sperm face repeated opportunities for fertilization. As *C. elegans* sperm are the rate limiting vehicles of heredity, and can cause pathology if unregulated, the ability to regulate sperm behavior and motility confers a massive fitness benefit. We still know very little about the mechanisms *C. elegans* sperm use to respond to their environment and ensure that every sperm gets an oocyte, but SPE-6 appears to play a central role.

## **Materials and Methods:**

### ***C. elegans* genetics and culturing:**

*C. elegans* genetics and husbandry were accomplished using standard methods (Brenner, 1974). With the exception of the biochemical experiments below, worms were cultured on MYOB plates seeded with OP50 strain of *E. coli* and maintained at 22°C unless otherwise noted. Some *C. elegans* strains were acquired from the Caenorhabditis Genetics Center. Strains used were all derived from N2 bristol and were as follows: CB1489 *him-8(e1489)* IV. *spe-6(hc163)* *dpy-18(e364)* III. *spe-6(hc187)* III. BA606 *spe-6(hc49)* *unc-25(e156)* III; *eDp6* (III;f). *spe-8(hc50)* I. *spe-27(it110)* IV. AV322 *swm-1(me87)* *him-5(e1490)* V. *snf-10(hc193)*. *emb-27(g48ts)* II; *him-5(e1492)* V. *fer-1(hc1)* I; *him-5(e1490)* V. *fog-2(q71)* II. JK816 *fem-3(q20)* IV, *fem-3(e2006)* IV. *spe-46 (hc197)* I.

### **Immunocytology:**

Whole gonads were obtained with individual dissections, sperm spreads were obtained by applying pressure to greased cover slips (Golden et al. 2000). For methanol fixation, samples were flash frozen in liquid nitrogen and fixed in cold -20° C methanol for at least 1 day. Fixed specimens were incubated with primary and secondary antibodies for 1.5-2 hours. The following primary antibodies were used: mouse monoclonal anti-MSP (1:1500) (4D5, gift from D. Greenstein),

mouse monoclonal 1CB4 (1:150) (Provided by Steve L'Hernault) and anti-SPE-6 (1:250) (Yenzyme). SPE-6 antibody was generated by Yenzyme in rabbits against the following C-terminal peptide epitope (360-379): KSLSAEKSCTKNVETARTEK (Figure I-3 ) (Yenzyme). SPE-6 antisera was affinity purified using the peptide antigen (Yenzyme). The following affinity purified commercial secondary antibodies were used: Dylite labeled anti-mouse (1:100) (Jackson ImmunoResearch Laboratories), TRITC labeled anti-rabbit (1:800) (Jackson ImmunoResearch Laboratories). Images were taken in the linear range and levels were lightly adjusted on photoshop with care taken to avoid discarding pixel intensity information,

### **Sperm activation counts:**

Synchronized worms were maintained at the indicated temperature starting at an embryonic or L1 stage. Males were picked away from hermaphrodites at the L4 stage to ensure male celibacy. Two days after isolation, or one day after isolation at 25°C to reflect temperature dependent metabolic differences, males were dissected as above and prepared for indirect immunofluorescence against MSP. Slides were blind scored; each slide was scored for the number of worms exhibiting precocious activation based on the presence or absence of MSP positive pseudopods. Only individuals with a large (>50) field of spermatids/spermatozoa were scored. The benchmarks of 0-1%, 1-5%, and >5% precociously activated spermatozoa were used in scoring to make our system sensitive to any level of precocious activation.

**Worm collection for western blot:**

For western blot experiments requiring isolated males, 80 worms (except in Figure 1 which contained 300 males of each genotype and 300 feminized hermaphrodites) were collected in 20 $\mu$ L of M9 buffer (recipe) containing protease inhibitor (Roche 11 836 170 001) in the cap of an Eppendorf tube. Samples were flash frozen in liquid nitrogen and stored at -80°C.

**Western blotting**

Western blot analysis was performed on lysates obtained from flash frozen worm samples in M9 buffer with a protease inhibitor added (Roche). Frozen samples were homogenized with 20  $\mu$ L sample buffer (Laemmli Sample Buffer, Bio Rad) containing 5% beta-mercaptoethanol (MP Biomedicals) heated to 95°C, boiled for 5 minutes, and centrifuged for 3 minutes at 15,000 relative centrifugal force (rcf). SDS-PAGE was performed on lysates from 80-200 worms at 150V (Mini-Protean® TGX Gels, Bio Rad) and transferred to a polyvinylidene difluoride membrane using a semi-dry protocol (GE Healthcare). Membranes were blocked overnight with Tris buffered saline with Tween 20 (TBST; 0.2M Tris HCl pH 8.0, 1.5M NaCl, 0.1% Tween 20) containing 4% non-fat dry milk (Carnation).

After blocking, membranes were incubated with the appropriate antibody diluted in blocking buffer (4% milk (w/v) in 1X TBST) for two hours at room temperature. Following two hour antibody incubations, membranes were washed for 15

minutes six times in 1x TBST. The following primary antibodies and concentrations were used for western blotting: HRP conjugated (Abcam) anti-SPE-6 2698 rabbit polyclonal (Yenzyme) at a 1:10,000 dilution and anti-MSP mouse monoclonal 4D5 at a 1:50,000 dilution (Courtesy David Greenstein). MSP blots were probed with an HRP conjugated polyclonal goat-anti-mouse secondary at a 1:10,000 dilution (Jackson Laboratories). Antibodies were detected with enhanced chemiluminescence (Immobilon™ Western Chemiluminescent HRP Substrate, Millipore), exposed to x-ray film, and developed.

#### **Sperm activation assays:**

Celibate aged male worms were dissected on + slides in a 1:100 pronase EB solution with care made to attempt to get large visible “streaks” of spermatids to stick to the positively charged slides. Pronase efficacy was verified by DIC on wild type sperm during each independent experiment, and attempts were sometimes made to take DIC pictures at specific coordinates to match to later immunofluorescence images. Times given are the time since the last cut was made during dissection, so the times given are the minimum time that the sperm were sitting in dissection solution with a variation of up to approximately three minutes; note that this is a different, less precise methodology than that used in Shakes and Ward’s “seminal” 1989 paper.

To assess the localization of SPE-6 in male *in vivo* activated sperm, wild type young adult males were plated with L4 *fog-2(q71)* feminized hermaphrodites for 24 hours. These mated feminized hermaphrodites were dissected (with attempts made to lay out spermathecae) and flash frozen for immunofluorescence as above.

### **Sperm motility quantification:**

Male gonads from animals raised at 25° C were dissected on 22x22 uncharged glass slides with 8uL of dissecting solution. Dissected sperm were imaged with time lapse photo microscopy using DIC optics. Quantitative analysis of sperm motility was performed with the use of FIJI ImageJ manual tracker plug in, and data was analyzed with Microsoft Excel.

### **Phosphatase assay:**

Large cultures of *fem-3(gf)* worms grown on NA22 *E. coli* were raised on peptone containing plates at a restrictive 25°, washed into M9 buffer, and flash frozen in liquid nitrogen. Worm samples were lysed using HEPES lysis buffer. Total protein concentration was determined using a Nanodrop spectrophotometer. A Lambda protein phosphatase kit was used and separate 1x lysate-phosphatase reactions were conducted with identical concentrations of lysate but increasing concentrations of Lambda phosphatase in the recommended buffering conditions (New England Biolabs). Reaction took place under room temperature conditions

for 30 minutes, after which reactions were halted by 2x boiling SDS with BME sample buffer. Stabilized samples were then frozen for storage and reheated for western blots.

## **References:**

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